

Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) **EP 1 120 422 A1**

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:
01.08.2001 Bulletin 2001/31

(51) Int Cl.7: **C07F 9/655**, C12Q 1/42,
C12Q 1/34

(21) Application number: **01104599.4**

(22) Date of filing: **06.05.1994**

(84) Designated Contracting States:
**AT BE CH DE DK ES FR GB GR IE IT LI LU MC NL
PT SE**

(30) Priority: **07.05.1993 US 57903**
25.04.1994 US 231673

(62) Document number(s) of the earlier application(s) in
accordance with Art. 76 EPC:
94915887.7 / 0 649 417

(71) Applicant: **TROPIX, INC.**
Bedford, MA 01730-2314 (US)

(72) Inventors:
• **Bronstein, Irena**
Newton, MA 02158 (US)

• **Edwards, Brooks**
Cambridge, MA 02139 (US)
• **Sparks, Allison**
North Andover, MA 01845 (US)

(74) Representative: **Albrecht, Thomas, Dr. et al**
Kraus & Welsert,
Thomas-Wimmer-Ring 15
80539 München (DE)

Remarks:

This application was filed on 06 - 03 - 2001 as a
divisional application to the application mentioned
under INID code 62.

(54) **Improved chemiluminescent 1,2-dioxetanes**

(57) Novel 1,2-dioxetanes with improved chemiluminescent properties, such as signal intensity, S/N ratio, T_{1/2}, etc. are provided by spiroadamantyl 1,2-dioxetanes, wherein the remaining carbon atom of the ring bears an alkoxy, aryloxy, or arylalkoxy substituent, and either a phenyl or naphthyl ring, this aromatic ring bearing, at the meta position on the phenyl group, or a non-conjugated position on the naphthyl ring, an OX moiety wherein X is an enzyme-cleavable group, which when

removed from the dioxetane, leaves the oxyanion which decomposes with chemiluminescence, the aryl ring further bearing an electron-active substituent Z. The nature and placement of the Z substituent, at a position not adjacent the point of attachment to the dioxetane ring, strongly influences the properties of the dioxetane. Assays, as well as kits for the performance of those assays, include the dioxetane, an enzyme capable of cleaving the X group, and in certain cases, membranes and chemiluminescent enhancement agents.

EP 1 120 422 A1

BEST AVAILABLE COPY

Description

[0001] This application claims priority of U.S. Application Serial No. 08/_____, filed April 25, 1994 as Attorney Docket No. 4085-061-27 CIP and U.S. Application Serial No. 08/057,903, filed May 7, 1993.

BACKGROUND OF THE INVENTION**Field of the Invention:**

[0002] This invention pertains to chemiluminescent 1,2-dioxetane derivatives which can be enzymatically activated to decompose and, through decomposition, release light. The dioxetanes are particularly characterized by the presence of an aromatic (phenyl or naphthyl) ring bonded to the dioxetane, which ring bears a meta-substituted or disjoint enzymatically cleavable group, which when cleaved, leaves the phenoxyanion or naphthyloxyanion of the dioxetane, and, at the four or the five position in the case of the phenyl, for example, an electron donating or electron withdrawing group. By selecting the identity of the substituent at the four or five position (the Z moiety) particular aspects of the chemiluminescent properties of the dioxetane, including half life, quantum yield, S/N ratio, etc., can be altered.

Background of the Invention:

[0003] 1,2-dioxetane enzyme substrates have been well established as highly efficient chemiluminescent reporter molecules for use in enzyme immunoassays and nucleic acid probe assays of a wide variety of types. These assays provide a preferred alternative to conventional assays that rely on radioisotopes, fluorophores, complicated color shifting, secondary reactions and the like. Dioxetanes developed for this purpose include those disclosed in U.S. Patent 4,978,614 as well as U.S. Patent 5,112,960. U.S. Patent 4,978,614 discloses, among others, 3-(2'-spiroadamantane) 4-methoxy-4-(3"-phosphoryloxy)phenyl-1,2-dioxetane, which has received world-wide attention, and is commercially available under the trade name AMPPD. U.S. Patent 5,112,960, discloses compounds, wherein the adamantyl stabilizing ring is substituted, at either bridgehead position, with a variety of substituents, including hydroxy, halogen, and the like, which convert the otherwise static or passive adamantyl stabilizing group into an active group involved in the kinetics of decomposition of the dioxetane ring. Compounds of this type have similarly received international attention, giving a faster and stronger signal than AMPPD in many applications. CSPD is a spiroadamantyl phenylphosphate dioxetane bearing a chlorine substituent on the adamantyl group, and, like AMPPD, is available from Tropix, Inc. of Bedford, Mass.

[0004] Compounds of this type have been particularly developed for enhanced sensitivity in assays for the presence of analytes in concentrations as low as 10^{-12} M and lower. In certain applications, compounds of this type are used in conjunction with enhancers to detect analytes in concentration of 10^{-12} M or lower. These enhancement agents, which include natural and synthetic water-soluble macromolecules, are disclosed in detail in U.S. Patent 5,145,772. Preferred enhancement agents include water-soluble polymeric quaternary ammonium salts, such as poly(vinylbenzyltrimethylammonium chloride) (TMQ), poly(vinylbenzyltributylammonium chloride) (TBQ) and poly(vinylbenzyltrimethylammonium chloride) (BDMQ).

[0005] These enhancement agents improve the chemiluminescent signal of the dioxetane reporter molecules, apparently by providing a hydrophobic environment in which the dioxetane is sequestered. Water, an unavoidable aspect of most assays, due to the use of body fluids, is a natural "quencher" of the dioxetane chemiluminescence. The enhancement molecules apparently exclude water from the microenvironment in which the dioxetane molecules, or at least the excited state emitter species reside, resulting in enhanced chemiluminescence. Other effects associated with the enhancer-dioxetane interaction could also contribute to the chemiluminescence enhancement.

[0006] Additional advantages can be secured by the use of selected membranes, including nylon membranes and treated nitrocellulose, providing a similarly hydrophobic surface for membrane-based assays, and other membranes coated with the enhancer-type polymers described.

[0007] Nonetheless, it remains a general goal of the industry to improve the performance of these stabilized, chemiluminescent dioxetane reporter molecules, to improve the machine readability, sensitivity, and performance aspects of the immunoassays, dependent on the chemiluminescent signal released by the dioxetanes.

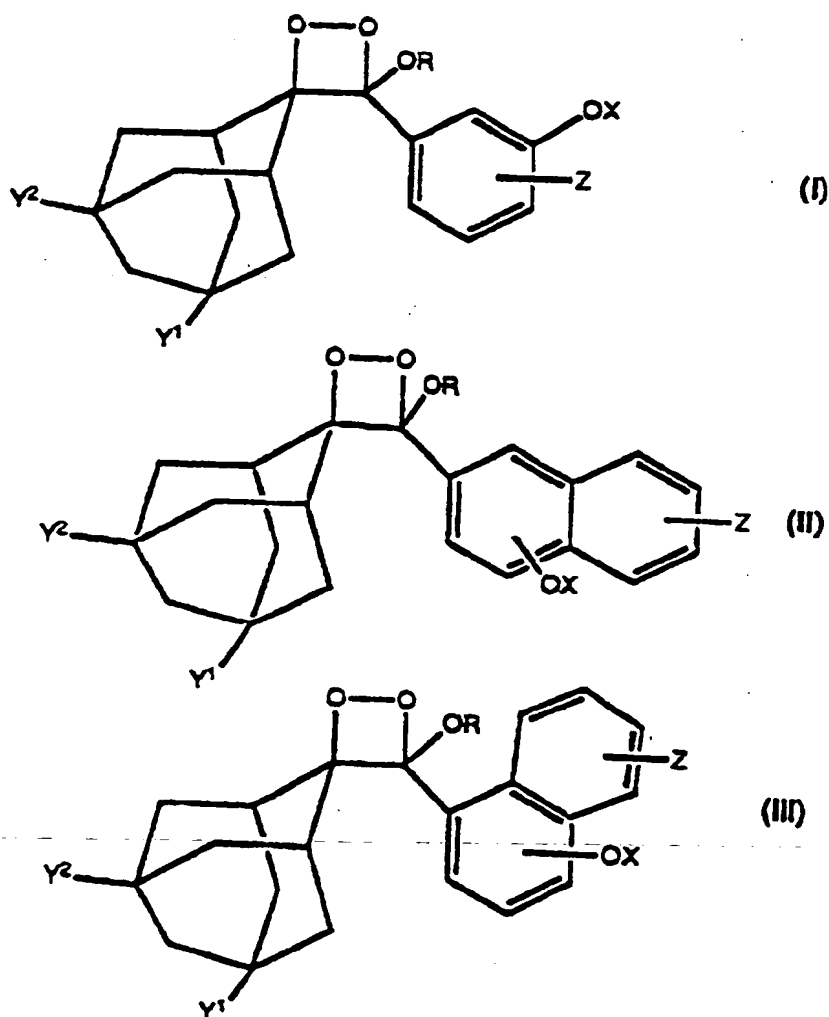
[0008] By way of background, and as disclosed in all the patents referenced above, the enzymatically-activated dioxetanes are used as reporter molecules, as substrates for enzymes which cleave the enzyme-labile group bonded to an aromatic substituent on the dioxetane ring. Thus, the enzyme, e.g., alkaline phosphatase is present alone or is covalently linked or otherwise complexed with either an antigen or antibody, in conventional antigen/antibody ligand binding assays, or a nucleic acid probe in nucleic acid assays. The enzyme-bearing antigen or antibody, or nucleic acid probe, is then admixed with the analyte suspected of containing the target antigen, or nucleic acid sequence, under conditions which permit complexing or hybridization between the antigen/antibody or probe/nucleic acid se-

quence. After washing away or separating off all noncomplexed or nonhybridized material, the dioxetane substrate is added. If the suspected analyte is present, the enzyme will cleave the enzyme-labile group on the aromatic substituent on the dioxetane, e.g., phenyl or naphthyl, yielding the phenoxy or naphthoxy anion intermediate. This anion decomposes, by electron transfer through the aromatic ring, cleaving the dioxetane ring, and yielding two carbonyl-based products. The cleavage/decomposition event is the light-releasing event.

[0009] To automate clinical assays, and to provide for substantial throughput, continued reductions in the half life, or $T_{1/2}$ of the dioxetane, as well as a reduction in the amount of time required to reach the maximum emission of light of the reporter molecule, is desirable. At the same time, to detect analytes in extremely low concentrations, below, e.g., about $10^{-12}M$, it is desirable to improve the intensity of the signal of the dioxetane reporter molecule, and simultaneously desirable to avoid increasing the background noise due to nonenzymatically-induced light release, so as to improve the overall sensitivity of the assay. Thus, further improvements in chemiluminescent dioxetane reporter molecules are sought.

SUMMARY OF THE INVENTION:

[0010] The above goals, and others, are met by a new class of dioxetanes, particularly characterized by a substituent on the aromatic ring bonded to the dioxetane, in addition to the meta-substituted enzyme-labile group. Thus, the novel dioxetanes of this invention have the generalized structure I, II or III below.



wherein R is C1-12 alkyl, aralkyl, or aryl, preferably C1-4 alkyl, X is an enzyme labile group cleavable by a specific enzyme which recognizes that group to leave the phenoxy or naphthoxy anion, and is preferably a phosphate, galac-

toside, or glucuronide. Y¹ and Y² are independently hydrogen, or an electron donating or withdrawing group, and are preferably hydrogen, methoxy, carboxy or halogen, and most preferably one of Y¹ and Y² is hydrogen while the other is chlorine, and Z is an electron-active group, most preferably chlorine, alkoxy, alkyl or amido. When Z is on a phenyl ring, Z is in the four or five position. When OX and Z are substituted on a naphthyl group, OX is substituted such that the substitution is disjoint, that is the total number of ring atoms between the point of attachment to the dioxetane ring and the point of substitution, including the point of attachment and substitution, is an odd number, as disclosed in U. S. Patent 4,952,707. Substituent Z may be substituted on the naphthyl ring at any position other than those adjacent the one position, or the point of attachment to the dioxetane ring.

[0011] By selecting the particular identity and location of Z, as an electron-withdrawing or an electron-donating group, specific characteristics of the chemiluminescent behavior of the dioxetane, including its $t_{1/2}$ -chemiluminescence half lives, time to maximum emission, maximum emission wavelength, and chemiluminescent signal intensity can be affected.

BRIEF DESCRIPTION OF THE DRAWINGS:

[0012] Figures 1 and 2 compare the performance of disodium 3(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo-[3.3.1.1^{3,7}]decan]-4-yl)phenyl phosphate dioxetane (CSPD) with a compound of this invention disodium-2-chloro-5-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro-)tricyclo-[3.3.1.1^{3,7}]decan]-4-yl)-phenyl phosphate where the phenyl moiety bears a chlorine substituent at the 4 position (CDP-Star). Figure 2 reflects the presence of a chemiluminescence enhancer, polyvinylbenzyltributylammonium chloride.

[0013] Figure 3 is a comparison between CSPD and CDP-Star on a nylon membrane assay for biotinylated pBR322-35mer.

[0014] Figures 4 and 5 are reproductions of Kodak XAR-5 film exposures of western blotting assays conducted on nylon and PVDF membranes comparing CSPD and CDP-Star.

[0015] Figures 6, 7 and 8 are reproductions of x-ray film contrasting CSPD and CDP-Star incubations of 10 minutes, 70 minutes and 19 hours, respectively, of an assay conducted on nylon membranes for the yeast gene RPB1.

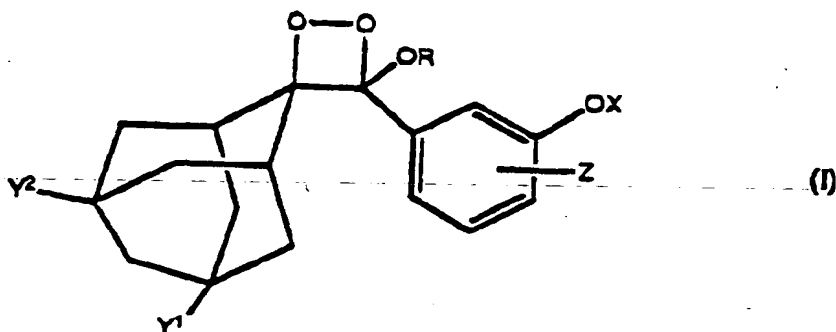
[0016] Figure 9 is a reproduction of x-ray film exposures reflecting chemiluminescent detection of DNA sequence ladders conducted on nylon membranes contrasting CSPD and CDP-Star.

[0017] Figures 10 and 11 are electrophotographic duplications of x-ray film images of DNA sequencing obtained by use of the dioxetanes of the claimed invention. These are compared against the current commercial standard, CSPD.

[0018] Figures 12-21 are electrophotographic duplications of dot blot assay results on membranes as indicated, employing dioxetanes of the claimed invention, dioxetanes outside the scope of the claimed invention and the commercial standards of CSPD and AMPPD. The membrane on which these assays were conducted is set forth in the Figures.

DETAILED DESCRIPTION OF THE INVENTION:

[0019] The dioxetanes of this invention are critically characterized by the substituents on the aromatic ring attached to the dioxetanes, which ring determines the electron transfer in the aryloxy anion, leading to decomposition and chemiluminescence. Thus, phenyl dioxetanes of the invention have the following and generalized structure (I).



[0020] Thus, the adamantyl-stabilized dioxetanes of the claimed invention bear two substituents on the phenyl ring in addition to the point of attachment of the dioxetane, as well as 0, 1 or 2 non-hydrogen substituents on the adamantyl ring. These substituents critically characterize the electronic characteristics of the dioxetane, the oxyanion, and its

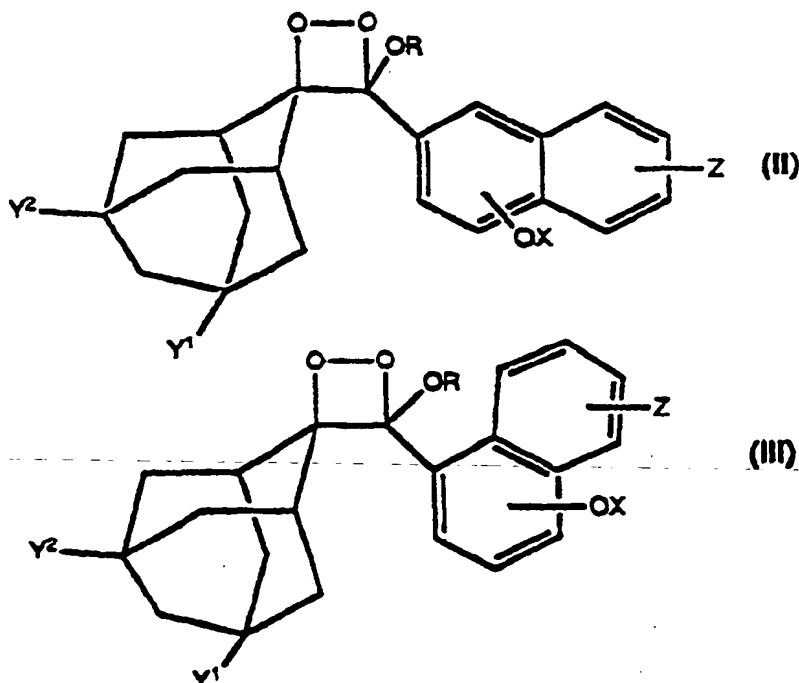
decomposition behavior. The identities of each substituent are set forth below.

[0021] R may be alkyl, aralkyl, cycloalkyl, or aryl, having 1-12 carbon atoms. R is preferably C1-C4 alkyl, more preferably C1-3 alkyl, most preferably methyl. The identity of R may be optimized with regard to solubility concerns, where unusual analytes, or buffers, may pose particular problems. Each of Y¹ and Y² represent, individually, and

[0022] X is an enzyme-cleavable moiety. Thus, upon proper contact with a suitable enzyme, X is cleaved from the molecule, leaving the oxygen attached to the phenyl ring, and thus, the phenoxy anion. X is ideally phosphate, galactoside, acetate, 1-phospho-2,3-diacylglyceride, 1-thio-D-glucoside, adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, adenosine, α -D-glucoside, β -D-glucoside, β -D-glucuronide, α -D-mannoside, β -D-mannoside, β -D-fructofuranoside, β -glucosiduronate, P-toluenesulfonyl-L-arginine ester, and P-toluenesulfonyl-L-arginine amide. X is preferably phosphate, galactoside or glucuronide, most preferably phosphate. It is important to note that when substituted on the phenyl ring, OX is meta with respect to the point of attachment to the dioxetane ring, that is, it occupies the three position.

[0023] Z may occupy either the four or five position. Z is an electron-active substituent, the character of the electron-active species (electron-donating or electron-withdrawing), optimizing various aspects of the dioxetane moiety. As an example, an electron-donating group, such as a methoxy group, may enhance the dioxetane phenoxy anion decomposition process, by facilitating the transferability of the free electrons from the aromatic ring O⁻ donor group, to the dioxetane ring. In contrast, an electron-withdrawing group would reduce or impair the ability to transfer the free electrons to the dioxetane, thus slowing the decomposition reaction and light emission, although ultimately giving a light signal of greater intensity. This should be contrasted with the impact of the electron-withdrawing substituent on the adamantyl group, such as chlorine, which substantially accelerates light emission, sharply reducing T_{1/2}. Of surprising significance is the fact that substitution in the six position is particularly undesirable. Such six-substituted phenyl dioxetanes exhibit extraordinarily fast decomposition kinetics, and nearly no light emission. While Applicants do not wish to be restricted to this theory, it is believed that this behavior is due to steric considerations, that is, the ortho substituent "turns" the phenyl ring such that it destabilizes the dioxetane ring (destabilization through steric forces, not electron transfer) and a substituent at the six position, e.g., methoxy, does not participate in electron transfer. As discussed below, experiments involving 6-substituted phenyl dioxetanes give essentially no signal.

[0024] The phenyl substituent on the dioxetane ring may instead be naphthyl (structures II and III) as



In the naphthyl dioxetane, identities for R, Y¹ and Y², X and Z remain the same. Instead of being restricted to the "meta" position, OX may occupy corresponding positions in the naphthyl ring, that is, non-conjugated positions, or positions such that the number of carbon atoms between the point of substitution and the point of attachment to the dioxetane ring, including the carbons at both point of attachment and point of substitution, are odd, as set forth in U.S. Patent 4,952,707. Phenyl meta-substituted dioxetanes, and naphthyl dioxetanes substituted according to the pattern described above, may generally be expected to give higher quantum yields than the corresponding para and conjugated systems.

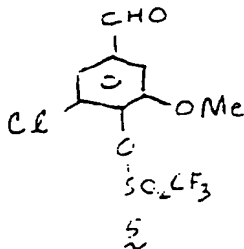
[0025] As noted above, Z can be any electron-active substituent that does not interfere with the chemiluminescent behavior of the dioxetane, and thus can be selected from a wide variety of identities. Preferred electron-active substituents include chloro, alkoxy (--OR), aryloxy (--OAr), trialkylammonium (--NR₃⁺), alkylamido (--NHCOR, --NRCOR'), arylamido (--NHCOAr, --NRCOAr, --NArCOAr), arylcarbamoyl (--NHCOOAr, --NRCOOAr), alkylcarbamoyl (--NHCOOR, --NRCOOR'), cyano (--CN), nitro (--NO₂), ester (--COOR, --COOAr), alkyl- or arylsulfonamido (--NHSO₂R, --NHSO₂Ar), trifluoromethyl (--CF₃), aryl (--Ar), alkyl (--R), trialkyl-, triaryl-, or alkylarylsilyl (--SiR₃, SiAr₃, --SiArR₂), alkyl- or arylamidodisulfonyl (--SO₂NHCOR, --SO₂NHCOAr), alkyl or aryl sulfonyl (--SO₂R, SO₂Ar) alkyl- or arylthioethers (--SR, SAR). The size of the Z substituent is generally limited only by solubility concerns. Where reference is made to alkyl or R, R', etc., the alkyl moiety should have 1-12 carbon atoms. Suitable aryl moieties include phenyl and naphthyl as exemplary moieties. Particularly preferred species include chloro and alkoxy.

[0026] Dioxetanes of the type described above, without the inclusion of the Z substituent, as previously noted, are disclosed in patents commonly assigned herewith. Patents addressing dioxetanes of this type without the inclusion of the Y and Z substituents have also been assigned to Wayne State University, such as 4,962,192. Substitution of the Z substituent on the dioxetanes required development of the synthesis of trisubstituted phenyl phosphonates which is described below, under the title Novel Tri-substituted Phenyl 1,2-Dioxetane Phosphates. The same general synthesis route can be employed for naphthyl dioxetanes embraced herein, bearing in mind the substitution patterns required, as discussed above. The synthesis of these compounds through the route described below involves the preparation of novel tri-substituted benzenes. Thus, as described below, an exemplary compound involved in the synthesis of the dioxetanes of this class includes 3-chloro-5-methoxybenzaldehyde. These tri-substituted compounds constitute key intermediates in a variety of synthetic pathways, the 1,3,5 substitution pattern being a generally preferred and widely applicable pattern. It is Applicants' belief that these intermediates have never previously been prepared, and are marked, in the synthesis route described below, with an asterisk.

NOVEL TRI-SUBSTITUTED PHENYL 1,2-DIOXETANE PHOSPHATES

Synthesis

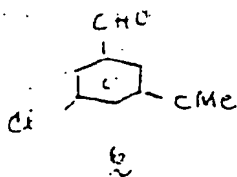
General. Commercial reagents were used as obtained without further purification. Baker silica gels (60-200 mesh for gram scale, and 230-400 mesh for milligram scale) were used for flash chromatography. ^{31}P NMR spectra were reported in parts per million relative to a phosphoric acid standard. High resolution mass spectral analyses were run by J.L. Kachinski at Johns Hopkins University. Syntheses of dioxetanes 3 and 4 were carried out following the procedure described below for dioxetanes 1 and 2 respectively. Yields, melting points (uncorrected) and spectral data are summarized for isolated intermediates.



3-Chloro-5-methoxy-4-trifluoromethanesulfonyloxy benzaldehyde (5). A solution of 5-Cl-vanillin¹ (13.0 g, 70 mmol), chloroform (4 ml) and pyridine (16 ml) was stirred at 0°C. Addition of trifluoromethanesulfonic anhydride (12.4 ml, 75 mmol) at 0°C over 30 min gave clean formation of the triflate. The reaction mixture was partitioned between EtOAc and 3N HCl, washed with dilute brine, dried over Na_2SO_4 , and evaporated under reduced pressure. Purification of the resulting yellow oil by silica gel chromatography (30% EtOAc/hexanes) yielded 18.5 g (83%) triflate 5 as yellow crystals.

IR (CHCl_3 , cm^{-1}): 1705, 1590, 1461, 1425, 1225, 1205, 1132, 1049, 875, 624

^1H NMR (ppm): 3.99 (3H, s), 7.44 (1H, d, $J=1.6$ Hz), 7.57 (1H, d, $J=1.7$ Hz), 9.92 (1H, s)

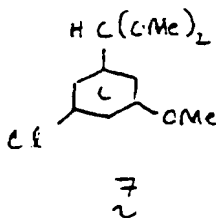


*** 3-Chloro-5-methoxybenzaldehyde (6).** Triflate 5 (9 g, 28 mmol), palladium(II) acetate (120 mg, 0.5 mmol), 1,1'-bis(diphenylphosphino)ferrocene (620 mg, 1 mmol) and hplc grade CH_3CN (10 ml) were mixed well in a teflon-lined stainless steel bomb. After adding freshly made, pulverized proton sponge formate² (7.84 g, 30 mmol), the bomb was sealed and heated at 90°C for 4 h. The cooled reaction was then filtered to remove proton sponge crystals, partitioned between EtOAc and 3N HCl, washed once each with dilute brine and dilute NaHCO_3 , dried over Na_2SO_4 , and evaporated. Silica gel chromatography (15% EtOAc/hexanes) yielded 4.25 g (88.5%) of chloromethoxybenzaldehyde 6, mp 45°C.

IR (CHCl_3 , cm^{-1}): 2835, 1700 ($\text{C}=\text{O}$), 1590, 1576, 1461, 1425, 1380, 1320, 1280, 1265, 1144, 1050, 850, 695

^1H NMR (ppm): 3.84 (3H, s), 7.13 (1H, m), 7.26 (1H, m), 7.41 (1H, m), 9.89 (1H, s)

Mass spectrum (EI, 70 eV): exact mass calcd for $\text{C}_8\text{H}_7\text{ClO}_2$ 170.0135, found 170.0134.

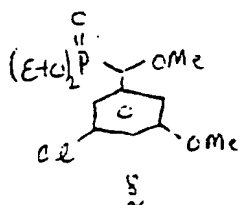


3-Chloro-5-methoxybenzaldehyde dimethyl acetal (7). A methanol solution (20 ml) of benzaldehyde 6 (8.76 g, 51 mmol) was cleanly converted to dimethyl

acetal 7 in the presence of trimethyl orthoformate (5.62 ml, 51 mmol) and a catalytic amount of p-toluenesulfonic acid. The reaction was quenched with triethylamine to pH 7, evaporated to a small volume and partitioned between EtOAc and NaHCO₃. The organic layer was dried, evaporated under reduced pressure and purified by silica gel chromatography (10% EtOAc/hexanes) to give 10.68 g (96%) of acetal 7 as a light yellow oil.

IR (CHCl₃, cm⁻¹): 2960, 2938, 2830, 1596, 1578, 1458, 1270, 1104, 1050, 989, 872, 865, 840

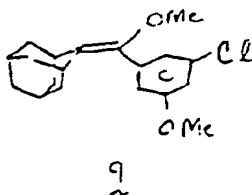
¹H NMR (ppm): 3.31 (6H, s), 3.79 (3H, s), 5.31 (1H, s), 6.85 (1H, s), 6.88 (1H, s), 7.04 (1H, s)



Diethyl 1-methoxy-1-(3-chloro-5-methoxyphenyl)methane phosphonate 8. Triethyl phosphite (3.2 ml, 19 mmol) was added dropwise to a solution of acetal 7 (4.0 g, 18.5 mmol), boron trifluoride etherate (2.3 ml, 19 mmol) and CH₂Cl₂ (20 ml) at 0°C. After slowly warming the reaction to room temperature (30 min), the solution was partitioned with dilute NaHCO₃, dried over Na₂SO₄, evaporated and purified on silica gel (40%-100% EtOAc/hexanes) to give 4.6 g (77.5%) of phosphonate 8 as a light yellow oil.

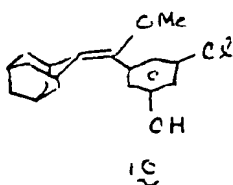
IR (CHCl₃, cm⁻¹): 2990, 1591, 1573, 1458, 1254 (P=O), 1050 (P-O), 1025 (P-O), 969, 870, 687

¹H NMR (ppm): 1.24 (3H, t, J=7 Hz), 1.26 (3H, t, J=7 Hz), 3.37 (3H, s), 3.78 (3H, s), 4.01-4.09 (4H, m), 4.40 (1H, d, J=16 Hz), 6.83 (1H, t, J=2 Hz), 6.88 (1H, qt, J=2 Hz), 6.98 (1H, qt, J=2 Hz)



3-Chloro-5-methoxy-1-(methoxycyclo[3.3.1]dec-2-ylidene)methylbenzene (9). Phosphonate 8 (4.62 g, 14 mmol) and 2-adamantanone (2.58 g, 17 mmol) were dissolved in anhydrous THF (35 ml) under argon and cooled to -68°C. Dropwise addition of lithium diisopropylamide (18.6 mmol) in anhydrous THF (20 ml) at -68°C generated the ylid, followed by subsequent olefination of the ketone. The reaction was slowly warmed to room temperature over 2 h and then stirred at 75°C for 1 h. The solution was partitioned between EtOAc/NH₄Cl, dried over Na₂SO₄, evaporated and purified by silica gel chromatography (2% EtOAc/hexanes), yielding 2.5 g (55%) of enol ether 9 as an oil.

¹H NMR (ppm): 1.55-1.95 (12H, m), 2.61 (1H, br s), 3.21 (1H, br s), 3.28 (3H, s), 3.78 (3H, s), 6.74 (1H, s), 6.80 (1H, s), 6.87 (1H, s)

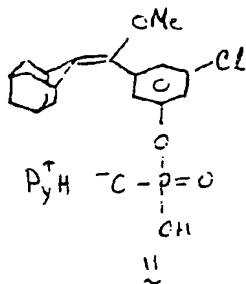


3-Chloro-5-hydroxy-1-(methoxycyclo[3.3.1]dec-2-ylidene)methylbenzene (10). Demethylation to enol ether phenol 10 proceeded cleanly upon heating enol ether 9 (2.5 g, 7.8 mmol) in DMF (14 ml) at 155°C in the presence of sodium ethane thiolate (11.7 mmol). Upon cooling, the mixture was partitioned between EtOAc and NH₄Cl, dried over Na₂SO₄ and evaporated under high vacuum to remove residual DMF. Chromatographic purification (silica gel, 20% EtOAc/hexanes) produced 2.3 g (96%) of phenol 10 as an oil which

crystallized upon standing. Trituration of the solid with 5% EtOAc/hexanes gave white crystals, mp 133°C.

IR (CHCl₃, cm⁻¹): 3584 (OH), 3300 (OH), 2910, 1590, 1310, 1285, 1163, 1096, 1080, 1011, 900, 840

¹H NMR (ppm): 1.73-1.96 (12H, m), 2.62 (1H, br s), 3.20 (1H, br s), 3.32 (3H, s), 5.65 (1H, br s), 6.73 (1H, s), 6.79 (1H, m), 6.85 (1H, s)



Pyridinium 3-chloro-5-(methoxytricyclo[3.3.1.1.3]dec-2-ylidenemethyl)-1-phenyl phosphate (11). Triethylamine (450 µl, 3.2 mmol) was added under an argon atmosphere to enol ether 10 (709 mg, 2.3 mmol) dissolved in anhydrous THF (10 ml). The solution was cooled to 0°C, at which time 2-chloro-2-oxo-1,3,2-dioxaphospholane (Fluka, 285 µl, 3.0 mmol) was added dropwise. The reaction was warmed to room temperature, quickly passed through an argon-flushed column under inert atmosphere to remove triethylammonium hydrochloride crystals. After rinsing the crystal cake once with THF, the solution was evaporated and pumped dry to give crude phospholane 11a.

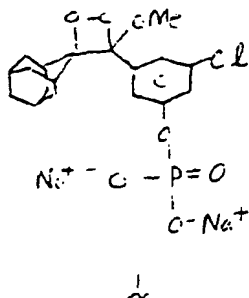
Opening the phospholane ring upon reaction of 11a with NaCN (vacuum dried, 179 mg, 3.65 mmol) in anhydrous DMF (6 ml) under argon, produced the desired β-cyanoethyl diester phosphate 11b, as well as regenerating enol ether phenol 10. Removal of DMF under high vacuum while warming the flask to 55°C, left a mixture of compounds 10 and 11b as a yellow-orange oil.

The above mixture was dissolved in methanol (8 ml) and stirred at 40°C in the presence of NaOMe (1 ml of 4.25 M NaOMe/MeOH, 6.4 mmol), effecting β-elimination of the cyanoethyl group to give enol ether phosphate 11 as the disodium salt. After evaporating the methanol, the solid was dissolved in water and partitioned with minimal EtOAc to recover phenol 10 (333 mg). Purification of the aqueous phase by preparative HPLC, using a CH₃CN/H₂O gradient through a polystyrene column (PLRP-S, Polymer Laboratories), followed by ion exchange with pyridinium toluenesulfonate (Amberlyst-IR 120+ resin) and lyophilization, yielded 448 mg (78% over 3 steps, accounting for recovered phenol) of enol ether phosphate 11 as a fluffy, off-white powder.

IR (CHCl₃, cm⁻¹): 2910, 1590, 1567, 1278, 1160, 1095, 945

¹H NMR (ppm): 1.73-1.96 (12H, m), 2.63 (1H, br s), 3.20 (1H, br s), 3.32 (3H, s), 5.89 (1H, s), 6.72 (1H, m), 6.79 (1H, t, J=2 Hz), 6.85 (1H, d, J=2 Hz)

³¹P NMR (ppm): 54 (1P)

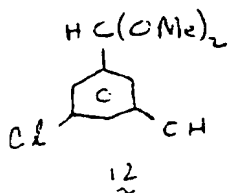


Disodium 3-chloro-5-(methoxyspiro[1.2-dioxetane-3,2'-tricyclo[3.3.1.1.3]decane]-4-yl)-1-phenyl phosphate (1). A solution of enol ether phosphate 11 and 5,10,15,20-tetraphenyl-21H,23H-porphine (TPP, 0.5 ml of a 2% solution in CHCl₃ by weight) in CHCl₃ (8 ml) was irradiated with a 250W, high pressure sodium lamp at 10°C while passing a stream of oxygen through the solution. A 5-mil

piece of Kapton polyimide film (DuPont) placed between the lamp and the reaction mixture filtered out unwanted UV radiation. Analytical HPLC (UV detector at 270 nm) showed complete dioxetane formation upon irradiating 5 min. After evaporation of the chloroform at 0°C, the residue was dissolved in ice water in the presence of Na₂CO₃ (27 mg, 0.25 mmol) and purified by preparative HPLC as described above. The fractions were frozen and lyophilized at 0°C, yielding 65.3 mg (90%) of dioxetane 1 as a fluffy white powder. TLC of the dioxetane exhibited blue chemiluminescence by thermal decomposition upon heating. Enzymatic cleavage of the phosphate also induced chemiluminescent decomposition in aqueous solutions.

¹H NMR (D₂O, ppm): 0.93 (1H, d, J=13 Hz), 1.21 (1H, d, J=13 Hz), 1.44-1.69 (10H, m), 2.16 (1H, br s), 2.78 (1H, br s), 3.14 (3H, s), 7.20 (2H, br s), 7.30 (1H, s)

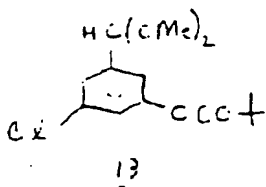
³¹P NMR (D₂O, ppm): 24 (1P)



3-Chloro-5-hydroxy benzaldehyde dimethyl acetal (12). 5-Chloro-3-methoxy benzaldehyde dimethyl acetal (7, 3.21 g, 14.8 mmol) was demethylated with sodium ethane thiolate (19 mmol) in DMF (14 ml) while heating at 150°C. The resultant phenol 12 was cooled, partitioned between EtOAc and NH₄Cl, dried over Na₂SO₄, evaporated and pumped to dryness on high vacuum to remove residual DMF. Chromatographic purification (silica gel, 20% EtOAc/hexanes) afforded 2.75 g (92%) of phenol 12 as a yellow oil. An analytical sample of the oil crystallized upon further purification, mp 153°C.

IR (CHCl₃, cm⁻¹): 3580 (OH), 3325 (OH), 2940, 2830, 1599, 1585, 1449, 1350, 1155, 1105, 1055, 894, 845

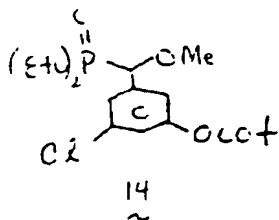
¹H NMR (ppm): 3.32 (6H, s), 5.30 (1H, s), 5.73 (1H, br s), 6.81 (2H, m), 7.01 (1H, s)



3-Chloro-5-pivaloyloxypbenzaldehyde dimethyl acetal (13). Phenol 12 (2.7 g, 13.3 mmol) and triethylamine (2.8 ml, 20 mmol) in CH₂Cl₂ (20 ml) were stirred at 0°C. Addition of trimethylacetyl chloride (1.64 ml, 13.3 mmol) cleanly yielded the pivaloyl ester. Standard workup provided crude pivaloate 13 as an oil which was carried on to the next reaction without purification; no weight was taken. A small sample was purified by prep TLC for spectral characterization.

IR (CHCl₃, cm⁻¹): 2980, 2940, 1749 (C=O), 1585, 1448, 1349, 1250, 1150, 1109, 1056, 898

¹H NMR (ppm): 1.34 (9H, s), 3.31 (6H, s), 5.36 (1H, s), 7.06 (2H, br s), 7.31 (1H, s)

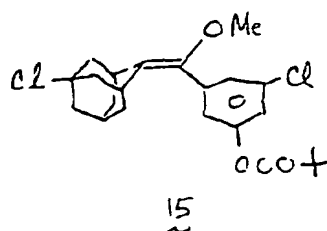


Diethyl 1-methoxy-1-(3-chloro-5-pivaloyloxypbenzyl)methane phosphonate (14). A solution of acetal 13, boron trifluoride etherate (2.6 ml, 21 mmol) and CH₂Cl₂ (10 ml) was stirred at -78°C. Addition of triethyl phosphite (3.0 ml, 17.5 mmol)

converted the acetal to phosphonate 14. Workup and purification (silica gel, 10% EtOAc/hexanes) yielded 2.43 g oil (47% over 2 steps).

IR (CHCl₃, cm⁻¹): 2995, 2980, 1750 (C=O), 1600, 1581, 1442, 1247 (P=O), 1110, 1028 (P-O), 975, 890

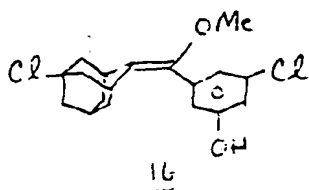
¹H NMR (ppm): 1.22-1.26 (6H, d of t, J=2 Hz, 7 Hz), 1.31 (9H, s), 3.39 (3H, s), 4.02-4.08 (4H, m), 4.44 (1H, d, J=16 Hz), 7.04 (2H, m), 7.27 (1H, br s)



3-Chloro-5-pivaloyloxy-1-(methoxy-5-chloro-tricyclo[3.3.1.1.3,7]-dec-2-ylidenemethyl)benzene (15). Phosphonate 14 (2.4 g, 6.1 mmol) was dissolved in anhydrous THF (10 ml) under argon and cooled to -68°C. Dropwise addition of lithium diisopropylamide (6.6 mmol) in anhydrous THF (7 ml) at low temperature generated the ylid, evident by deep coloration. After 5 min, a THF solution of 5-chloro-2-adamantanone (941 mg, 5 mmol) was added and the reaction was slowly warmed to room temperature over 40 min, followed by heating at 75° for 1 h to complete olefination. The solution was partitioned between EtOAc/NH₄Cl, dried over Na₂SO₄ and evaporated to give a crude mixture of enol ether pivaloate 15 and the corresponding enol ether phenol 16. The crude oil was used without purification in the following hydrolysis. A small sample was purified by prep TLC for spectral characterization.

IR (CHCl₃, cm⁻¹): 2935, 1750 (C=O), 1595, 1571, 1450, 1420, 1397, 1275, 1160, 1110, 1024, 918, 906, 887, 829

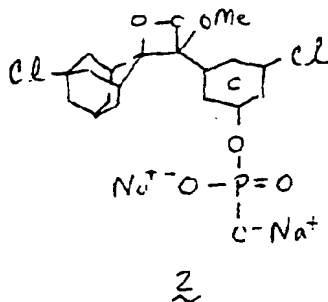
¹H NMR (ppm): 1.34 (9H, s), 1.68-1.78 (4H, m), 2.14-2.25 (7H, m), 2.77 (1H, br s), 3.30 (3H, s), 3.42 (1H, br s), 6.88 (1H, d, J=1.5 Hz), 7.04 (1H, m), 7.11 (1H, d, J=1.5 Hz)



3-Chloro-5-hydroxy-1-(methoxy-5-chloro-tricyclo[3.3.1.1.3,7]-dec-2-ylidenemethyl)benzene (16). Crude pivaloate 15 was hydrolyzed at room temperature with K₂CO₃ (1.45 g, 10.5 mmol) in 10 ml methanol. Evaporation of methanol, followed by standard workup and purification (silica gel, 30% EtOAc/hexanes) afforded 1.095 g (63% over 2 steps) of a slightly yellow oil which solidified upon standing. Titration of the solid produced white crystalline enol ether phenol 16, mp 130°C.

IR (CHCl₃, cm⁻¹): 3590 (OH), 3300 (OH), 2935, 1595, 1163, 1100, 1082, 1030, 911

¹H NMR (ppm): 1.69-1.83 (4H, m), 2.14-2.27 (7H, m), 2.77 (1H, br s), 3.30 (3H, s), 3.41 (1H, br s), 5.21 (1H, br s), 6.67 (1H, d, J=1.5 Hz), 6.81 (1H, m), 6.84 (1H, d)



Disodium 3-chloro-5-(methoxyspiro[1.2-dioxetane-3,2'-(5-chloro)-tricyclo[3.3.1.1.3,7]-dec-1-yl]-1-phenyl phosphonate (2). Triethylamine (230 µl, 1.65 mmol) was added under an argon atmosphere to enol ether 16 (356 mg, 1.05 mmol) dissolved in anhydrous THF (5 ml). The solution was cooled to 0°C, at which time 2-chloro-2-oxo-1,3,2-dioxaphospholane (Fluka, 143 µl, 1.55 mmol)

was added dropwise. The reaction was warmed to room temperature and quickly passed through an argon-flushed column under inert atmosphere to remove triethylammonium hydrochloride crystals. After rinsing the crystal cake once with THF, the solution was evaporated and pumped dry to give crude phospholane 17a.

Opening the phospholane ring upon reaction with NaCN (vacuum dried, 69 mg, 1.4 mmol) in anhydrous DMF (5 ml) under argon, produced the desired β -cyanoethyl diester phosphate 17b. Removal of DMF under high vacuum while warming the flask to 55°C left the crude diester phosphate as an orange oil.

A solution of cyanoethyl phosphate 17b and 5,10,15,20-tetraphenyl-21H,23H-porphine (TPP, 1.5 ml of a 2% solution in CHCl_3 by weight) in CHCl_3 (10 ml) was irradiated with a 250W, high pressure sodium lamp at 10°C while passing a stream of oxygen through the solution. A 5-mil piece of Kapton polyimide film (DuPont) placed between the lamp and the reaction mixture filtered out unwanted UV radiation. Analytical HPLC (UV detector at 270 nm) showed complete dioxetane formation upon irradiating 15 min. After evaporation of the chloroform at 0°C, the residue was dissolved in methanol and deprotected to the disodium phosphate dioxetane with NaOMe (0.5 ml of 4.25 M NaOMe/MeOH, 2 mmol). Upon β -elimination of the cyanoethyl group, the solvent was evaporated at 0° and the residue dissolved in ice water. Purification by preparative HPLC, as described above, followed by lyophilization at 0°C, yielded 289 mg (60% over 4 steps) of dioxetane 2 as a fluffy white powder.

^1H NMR (D_2O , ppm, mixture of syn/anti isomers): 0.86 (1H, d), 1.13 (1H, d, $J=14$ Hz), 1.30 (1H, d), 1.37 (1H, d), 1.45-2.07 (18H, m), 2.27 (1H, br s), 2.32 (1H, br s), 2.95 (2H, br s), 3.09 (3H, s), 3.11 (3H, s), 7.0-7.3 (4H, br s), 7.25 (1H, s), 7.28 (1H, s).

3,5-Dimethoxybenzaldehyde dimethyl acetal (18).

IR (CHCl_3 , cm^{-1}): 2958, 2935, 1598, 1460, 1426, 1357, 1190, 1154, 1101, 1053, 840

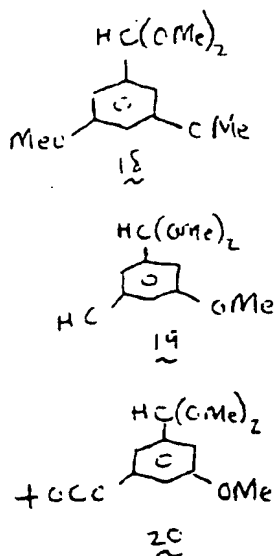
^1H NMR (ppm): 3.32 (6H, s), 3.78 (6H, s), 5.28 (1H, s), 6.41 (1H, m), 6.60 (2H, m)

3-Hydroxy-5-methoxybenzaldehyde dimethyl acetal (19).

IR (CHCl_3 , cm^{-1}): 3590 (OH), 3345 (OH), 2940, 2830, 1600, 1462, 1432, 1355, 1190, 1150, 1110, 1055, 841

^1H NMR (ppm): 3.32 (6H, s), 3.77 (3H, s), 5.28 (1H, s), 6.37 (1H, d, $J=2$ Hz), 6.53 (1H, br s), 6.58 (1H, br s)

3-Methoxy-5-pivaloxybenzaldehyde dimethyl acetal (20). (73% over 3 steps, oil)



IR (CHCl₃, cm⁻¹): 2960, 2935, 1741 (C=O), 1608, 1597, 1462, 1350, 1273, 1190, 1139, 1115, 1056, 999, 902, 848

¹H NMR (ppm): 1.34 (9H, s), 3.31 (6H, s), 3.80 (3H, s), 5.35 (1H, s), 6.57 (1H, d, J=2 Hz), 6.75 (1H, br s), 6.87 (1H, br s)

Diethyl 1-methoxy-1-(3-methoxy-5-pivaloyloxypheyl)methane phosphonate (21). (40%, oil)

IR (CHCl₃, cm⁻¹): 2990, 2980, 1742 (C=O), 1606, 1590, 1463, 1272, 1240, 1136, 1110, 1100, 1055, 1023, 970

¹H NMR (ppm): 1.21 (3H, t, J=3 Hz), 1.23 (3H, t), 1.32 (9H, s), 3.39 (3H, s), 3.78 (3H, s), 4.06 (4H, m), 4.44 (1H, d, J=16 Hz), 6.56 (1H, m), 6.72 (1H, m), 6.85 (1H, m)

3-Methoxy-5-pivaloyloxy-1-(methoxytricyclo[3.3.1.1^{3,7}]dec-2-ylidenemethyl)-benzene (22a)

IR (CHCl₃, cm⁻¹): 2910, 1740 (C=O), 1600, 1580, 1460, 1325, 1272, 1140, 1114, 1097, 1079, 1055

¹H NMR (ppm): 1.35 (9H, s), 1.56-1.96 (12H, m), 2.68 (1H, br s), 3.23 (1H, br s), 3.31 (3H, s), 3.80 (3H, s), 6.53 (1H, t, J=2 Hz), 6.61 (1H, br s), 6.72 (1H, m)

3-Hydroxy-5-methoxy-1-(methoxytricyclo[3.3.1.1^{3,7}]dec-2-ylidenemethyl)-benzene (22). (64%, white crystals, mp 159°C)

IR (CHCl₃, cm⁻¹): 3590 (OH), 3320 (OH), 2910, 1591, 1342, 1150, 1098

¹H NMR (ppm): 1.78-1.97 (12H, m), 2.68 (1H, br s), 3.23 (1H, br s), 3.33 (3H, s), 3.78 (3H, s), 5.49 (1H, s), 6.37 (1H, m), 6.45 (2H, m)

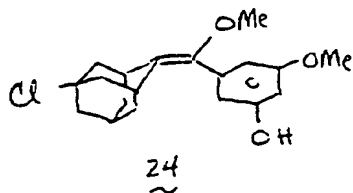
Pyridinium 5-methoxy-3-(methoxytricyclo[3.3.1.1^{3,7}]dec-2-ylidenemethyl)-1-phenyl phosphonate (23). (62%, off-white fluffy powder)

IR (CHCl₃, cm⁻¹): 2911, 1584, 1448, 1425, 1328, 1149, 1099, 960, 870

¹H NMR (ppm): 1.68-1.92 (12H, m), 2.63 (1H, br s), 3.17 (1H, br s), 3.23 (3H, s), 3.68 (3H, s), 6.55 (1H, br s), 6.72 (1H, br s), 6.76 (1H, br s), 6.98 (1H, br s)

Disodium 5-methoxy-3-(methoxyspiro[1.2-dioxerane-3,2'-tricyclo[3.3.1.1^{3,7}]decan]-4-yl)-1-phenyl phosphonate (3). (85%, white fluffy powder)

¹H NMR (D₂O, ppm): 0.98 (1H, br d), 1.22 (1H, br d), 1.46-1.76 (10H, m), 2.20 (1H, br s), 2.78 (1H, br s), 3.14 (3H, s), 3.74 (3H, s), 6.91 (1H, br s), 6.68-6.97 (2H, very broad signal)

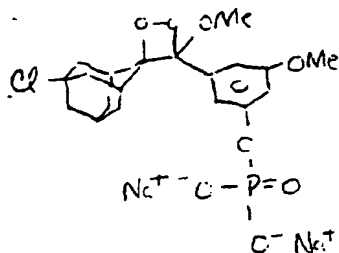


^{31}P NMR (D_2O , ppm): 44.8 (1P)

3-Hydroxy-5-methoxy-1-(methoxy-5-chloro-tricyclo[3.3.1.1.3.7]-dec-2-ylidene)methylbenzene (24). (63%, white crystals, mp 134°C)

IR (CHCl_3 , cm^{-1}): 3590 (OH), 3330 (OH), 2930, 1610, 1591, 1450, 1430, 1341, 1150, 1100, 1080, 1056, 1028, 829

^1H NMR (ppm): 1.68-2.40 (11H, m), 2.82 (1H, br s), 3.31 (3H, s), 3.42 (1H, br s), 3.78 (3H, s), 6.37-6.41 (3H, m)



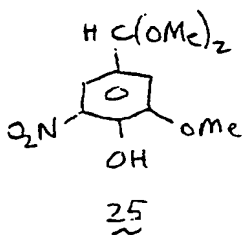
Disodium 5-methoxy-3-(methoxyspiro[1.2-dioxetane-3,2'-(5-chloro-tricyclo[3.3.1.1.3.7]-decan)-4-yl]-1-phenyl phosphate (4). (57% over 4 steps, white fluffy powder)

^1H NMR (D_2O , ppm, mixture of syn/anti isomers): 0.94 (1H, br d), 1.19 (1H, br d), 1.42 (1H, br d), 1.50 (1H, br s), 1.58 (1H, br d), 1.67-2.16 (17H, m), 2.38 (1H, br s), 2.40 (1H, br s), 3.00 (2H, br s), 3.15 (3H, s), 3.16 (3H, s), 3.73 (3H, s), 3.74 (3H, s), 6.90 (1H, br s), 6.93 (1H, br s), 6.65-7.00 (4H, very broad signal)

^{31}P NMR (D_2O , ppm, mixture of syn/anti isomers): 44.8 (2P)

References

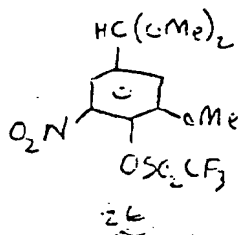
1. 5-Chlorovanillin was synthesized as described by Hann and Spencer (J. Am. Chem. Soc., 1927, 49:535-537), mp 163°C.
2. Proton sponge formate (N,N,N',N' -tetramethyl-1,8-naphthalenediammonium formate): Formic acid (98%, 1.2 ml, 31 mmol) was added to a solution of proton sponge (6.8 g, 32 mmol) and CH_2Cl_2 (8 ml) at 0°C. After warming to room temperature, the solvent was evaporated and the proton sponge formate crystallized as white crystals while drying on high vacuum with minimal warming. Proton sponge formate crystals (mp 79°C) must be used soon after preparation since formic acid will evaporate upon standing, leaving proton sponge (mp 50°C).



3-Methoxy-5-nitro-4-hydroxy benzaldehyde dimethyl acetal (25). A methanol solution (30 ml) of 5-nitrovanillin (5.0 g, 97%, 18.4 mmol) was cleanly converted to dimethyl acetal 25 in the presence of trimethyl orthoformate (2.8 ml, 25 mmol) and a catalytic amount of p-toluenesulfonic acid. The reaction was quenched with triethylamine to pH 8, evaporated to a small volume and partitioned between EtOAc and NaHCO₃. The aqueous layer was washed once with EtOAc. The organic layers were dried over Na₂SO₄, decanted and evaporated to an orange-red oil that crystallized upon pumping. Recrystallization from 50% EtOAc/hexanes gave 5.55 g (93%) acetal 25 as red-orange crystals, mp 58-59°C.

IR (CHCl₃, cm⁻¹): 3300, 3010, 2930, 2820, 1620, 1543, 1460, 1445, 1392, 1341, 1320, 1254, 1132, 1101, 1058, 990, 865

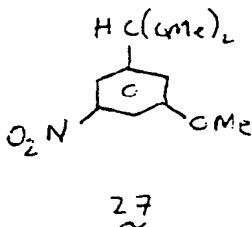
¹H NMR (ppm): 3.31 (6H, s), 3.94 (3H, s), 5.31 (1H, s), 7.22 (1H, d, J = 1.7 Hz), 7.78 (1H, d)



3-Methoxy-5-nitro-4-trifluoromethanesulfonyloxy benzaldehyde dimethyl acetal (26). A solution of dimethyl acetal 25 (5.0 g, 20.6 mmol), chloroform (3 ml) and pyridine (8 ml) was stirred at 0°C under argon. Addition of trifluoromethanesulfonic anhydride (4.0 ml, 23.8 mmol) at 0°C over 10 min, followed by stirring at room temperature overnight gave clean formation of the triflate. The solvents were evaporated under high vacuum while warming the oil to 45°C and traces of pyridine were chased with 4 ml toluene. The resulting oil was pumped well under high vacuum, taken up in 50% EtOAc/hexanes and titrated with 50% EtOAc/hexanes to separate the desired triflate (in solution) from the fine pyridinium triflate crystals. Evaporation of the titration solution, followed by purification of the oil on a silica gel column, eluting with 30% EtOAc/hexanes, yielded 6.43 g (84%) of triflate 26 as a light yellow oil.

IR (CHCl₃, cm⁻¹):

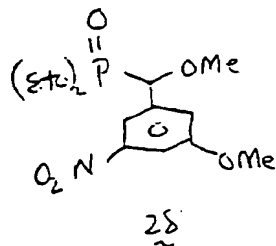
¹H NMR (ppm): 3.35 (6H, s), 4.00 (3H, s), 5.42 (1H, s), 7.42 (1H, d, J=1.6 Hz), 7.73 (1H, d)



→ **3-Methoxy-5-nitro-benzaldehyde dimethyl acetal (27).** 5-Nitrophenyl triflate 26 (7 g, 18.7 mmol), palladium (II) acetate (88 mg, 0.39 mmol), 1,1'-bis(diphenylphosphino)ferrocene (430 mg, 0.78 mmol) and hplc grade CH₃CN (10 ml) were mixed well in a teflon-lined stainless steel bomb. After adding freshly made, pulverized proton sponge formate (5.1 g, 19.6 mmol), the bomb was sealed and heated at 90°C for 2 h. The reaction mixture was taken up in EtOAc, passed through a silica gel plug, and then purified on a silica gel column, eluting with 0-30% EtOAc/hexanes to yield 1.5 g (35%) methoxynitrobenzaldehyde acetal 27.

IR (CHCl₃, cm⁻¹): 3005, 2960, 2935, 2835, 1532 (-NO₂), 1463, 1450, 1343 (-NO₂), 1280, 1190, 1158, 1104, 1055, 990, 871

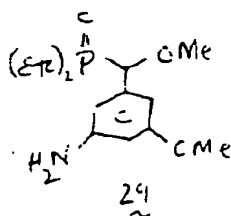
^1H NMR (ppm): 3.33 (6H, s), 3.89 (3H, s), 5.41 (1H, s), 7.33 (1H, s), 7.68 (1H, s), 7.92 (1H, s)



Diethyl 1-methoxy-1-(3-methoxy-5-nitrophenyl)methane phosphonate (28). Triethyl phosphite (0.98 ml, 5.7 mmol) was added dropwise to a solution of dimethyl acetal 27 (1.08 g, 4.7 mmol), boron trifluoride etherate (1.2 ml, 9.8 mmol) and CH_2Cl_2 (10 ml) at 0°C . After warming the reaction to room temperature overnight, the solution was partitioned with 3N HCl and the aqueous layer was washed with CH_2Cl_2 twice. The organic layers were washed with dilute NaHCO_3 , dried over Na_2SO_4 , decanted and evaporated. The crude residue was purified on a silica gel column, eluting with 0-80% EtOAc/hexanes, to give 1.36 g (86%) phosphonate 28 as a slightly yellow oil.

IR (CHCl_3 , cm^{-1}): 2995, 1532 ($-\text{NO}_2$), 1350 ($-\text{NO}_2$), 1280, 1258, 1243, 1096, 1053, 1025, 973, 721

^1H NMR (ppm): 1.28 (6H, t, $J = 7.1$ Hz), 3.44 (3H, s), 3.90 (3H, s), 4.08-4.15 (4H, m), 4.55 (1H, d, $J = 16$ Hz), 7.34 (1H, d), 7.69 (1H, d, $J = 2.1$ Hz), 7.87 (1H, d, $J = 1.6$ Hz)



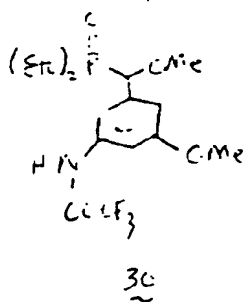
Diethyl 1-methoxy-1-(3-amino-5-methoxyphenyl)methane phosphonate (29).

Nitro phosphonate 28 is dissolved in methylene chloride and added to a 1M NaOH solution containing $n\text{Bu}_4\text{NBr}$ and sodium hydrosulfite. The biphasic solution is stirred vigorously, with warming if necessary, until reduction of the nitro substituent to aniline 29 is complete. The cooled solution is partitioned between CH_2Cl_2 and minimal water, and the aqueous layer is washed with CH_2Cl_2 as needed to obtain the crude aniline. The combined organic layers are dried, decanted and evaporated. The residue is then passed through a short silica gel plug to give aniline 29.

IR (CHCl_3 , cm^{-1}):

^1H NMR (ppm):

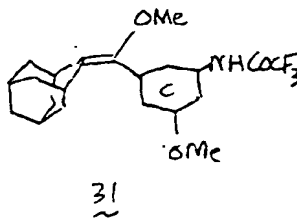
(References for other reduction conditions are appended to the synthesis summary.)



Diethyl 1-methoxy-1-(3-methoxy-5-trifluoroacetamidophenyl)methane phosphonate (30). Phosphonate 29 is quantitatively acetylated by addition of trifluoroacetic anhydride (1 eq) and triethylamine (1.3 eq) in 10 ml CH_2Cl_2 at 0°C . Evaporation of solvents, followed by silical gel column purification yields trifluoroacetamide 30.

IR (CHCl_3 , cm^{-1}):

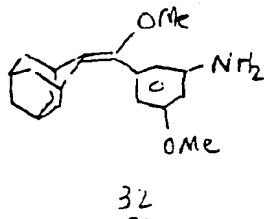
^1H NMR (ppm):



3-Methoxy-5-trifluoroacetamido-1-(methoxytricyclo[3.3.1.1.3.7]dec-2-ylidene-methyl)benzene (31). Phosphonate 30, dissolved in anhydrous THF, is cooled to -68°C under an argon atmosphere. Similarly, 2-adamantanone (1.1 eq) is dissolved in anhydrous THF and cooled to -68°C under argon in a separate flask. To the phosphonate solution is added 2.5M nBuLi at -68°C under argon until the red color of the ylid persists. At this point, 1.2 eq nBuLi is added to complete the ylid formation and the resulting colored solution is stirred at -68°C for 5 min. While maintaining the low temperature, 2-adamantanone in THF is slowly added to the ylid over an hour. After the final addition of ketone, the reaction mixture is stirred for 2 h while warming to room temperature. The reaction is then heated at reflux for 1 h, cooled and quenched by partitioning with EtOAc and saturated NH_4Cl . The organic layer is dried over Na_2SO_4 and chromatographed with EtOAc/hexanes on a silica gel column to give enol ether 31.

IR (CHCl_3 , cm^{-1}):

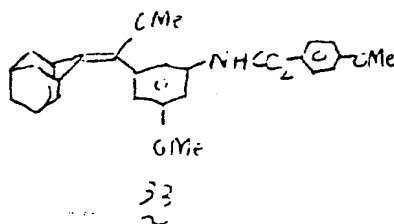
^1H NMR (ppm):



3-Amino-5-methoxy-1-(methoxytricyclo[3.3.1.1.3.7]dec-2-ylidenemethyl)benzene (32). Trifluoroacetamide enol ether 31 is hydrolyzed at 60°C with finely ground K_2CO_3 (3 eq) in MeOH containing trace water. Work up by partitioning the mixture with EtOAc/ H_2O , followed by silica gel chromatography provides enol ether aniline 32.

IR (CHCl_3 , cm^{-1}):

^1H NMR (ppm):

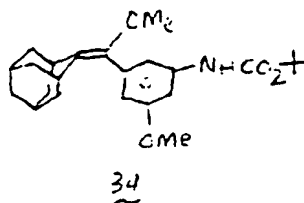


3-Carbamoyl-5-methoxy Derivatives (3-NHCO₂X):

3-para-Methoxyphenylcarbamoyl-5-methoxy-1-(methoxytricyclo[3.3.1.1.3.7]dec-2-ylidenemethyl)benzene (33). Enol ether aniline 32 in methylene chloride is carboxylated with 4-methoxyphenyl chloroformate (1.1 eq) in the presence of triethylamine (2.0 eq) at 0°C . The reaction mixture is partitioned with $\text{CH}_2\text{Cl}_2/\text{H}_2\text{O}$, washed with dilute NaHCO_3 , dried over Na_2SO_4 , evaporated and chromatographed on silica gel to yield enol ether *p*-methoxyphenylcarbamate 33.

IR (CHCl_3 , cm^{-1}):

^1H NMR (ppm):



3-tert-Butylcarbamoyl-5-methoxy-1-(methoxytricyclo[3.3.1.1.3.7]dec-2-ylidenemethyl)benzene (34). A methylene chloride solution of enol ether aniline 32, triethylamine (1.5 eq) and BOC-ON (1.3 eq) is stirred at 55°C in a tightly capped Kimax tube to effect *t*-butyl carbamate formation. The solution is cooled, evaporated to a small volume and, upon addition of MeOH to the residue, the desired carbamate 34 precipitates.

IR (CHCl₃, cm⁻¹):¹H NMR (ppm):3-N-Sulfonamido-5-methoxy Derivatives (3-NHSO₂X):

3-N-Toluenesulfonamido-5-methoxy-1-(methoxycyclo[3.3.1.1^{3,7}]dec-2-ylidenemethyl)benzene (35). A methylene chloride solution of enol ether aniline 32 is sulfonylated with tosyl chloride (1.1 eq) in the presence of triethylamine (2.0 eq) at 0°C. The reaction mixture is partitioned with CH₂Cl₂/H₂O, washed with dilute NaHCO₃, dried over Na₂SO₄, evaporated and chromatographed on silica gel to yield N-toluenesulfonamido enol ether 35.

IR (CHCl₃, cm⁻¹):¹H NMR (ppm):

3-N-Trifluoromethylsulfonamido-5-methoxy-1-(methoxycyclo[3.3.1.1^{3,7}]dec-2-ylidenemethyl)benzene (36). A methylene chloride solution of enol ether aniline 32 is sulfonylated with trifluoromethylsulfonic anhydride (1.1 eq) at 0°C. The reaction mixture is partitioned with CH₂Cl₂/H₂O, dried over Na₂SO₄, evaporated and chromatographed on silica gel to yield N-trifluoromethylsulfonamido enol ether 36.

IR (CHCl₃, cm⁻¹):¹H NMR (ppm):3-Amido-5-methoxy Derivatives (3-NHCOX):

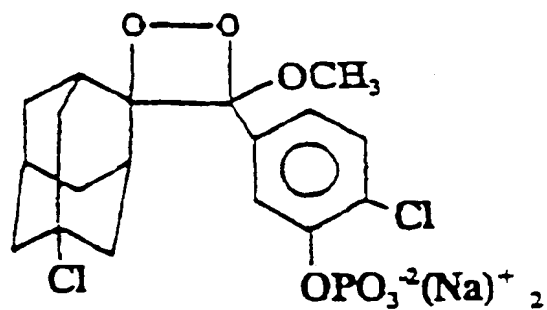
3-N-Benzamido-5-methoxy-1-(methoxycyclo[3.3.1.1^{3,7}]dec-2-ylidenemethyl)benzene (37). A pyridine solution of enol ether aniline 32 is reacted with benzoyl chloride (1.1 eq) at 0°C. The solvent is evaporated and pumped well to yield a crude oil, which is partitioned between CH₂Cl₂/H₂O, dried and evaporated. Chromatography on silica gel yields benzamido enol ether 37.

IR (CHCl₃, cm⁻¹):¹H NMR (ppm):

The 3-nitrogen-substituted phenyl enol ethers (compounds 33-37) are demethylated with sodium ethane thiolate, and then phosphorylated and

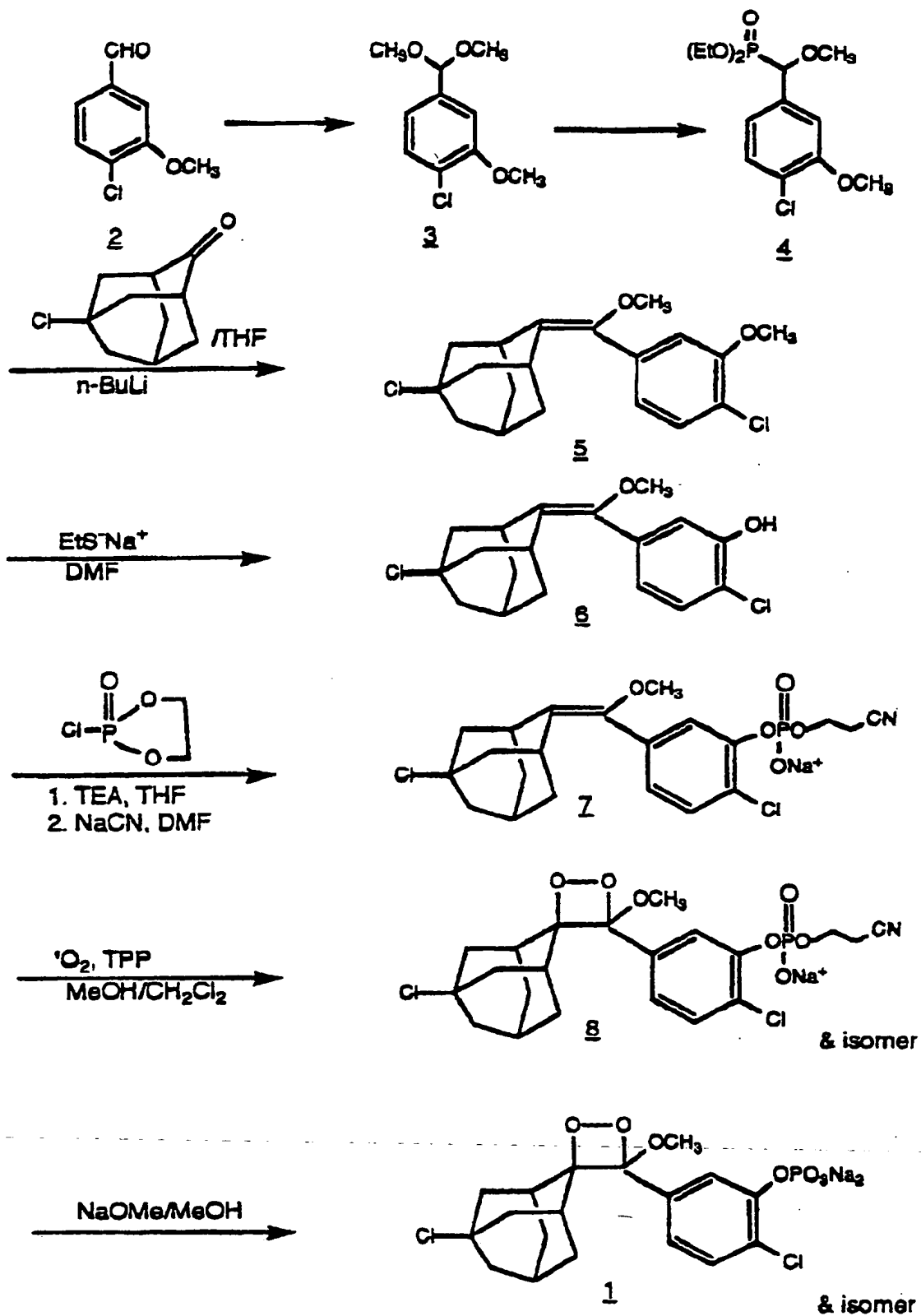
photooxygenated as described for dioxetanes 1 and 2 to obtain the analogous dioxetanes.

[0027] Among other inventive compounds within the structure of formula I, a particularly preferred compound has the structure:



[0028] The name of this compound is disodium 2-chloro-5-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro-)tricyclo[3,3,1.1^{3,7}]decan]-4-yl)-1-phenyl phosphate.

[0029] This compound is generally referred to as CDP-Star. It can be synthesized as shown in the following reaction scheme.



4-Chloro-3-methoxybenzaldehyde dimethyl acetal 3

[0030] A heterogeneous mixture of methanol (2 ml), CH_2Cl_2 (3 ml), 4-chloro-3-methoxybenzaldehyde (2 g, 11.7 mmol; prepared essentially as described by R.M. Riggs et al., J. Med. Chem., 30 1887, 1987.), trimethyl orthoformate (1.7 ml, 15.5 mmol) and a large crystal of p-toluenesulfonic acid was stirred at room temperature for one hour. Additional MeOH (1 ml) and a crystal of p-toluenesulfonic acid were added and the solution was warmed until homogeneous. Upon completion of the reaction, the solution was stirred 5 min with excess solid NaHCO_3 and rotary evaporated to remove solvents. The paste was dissolved in 40 ml EtOAc, partitioned against dilute NaHCO_3 solution, and evaporated to yield a light brown oil. The reaction was repeated with another 2 g of 4-chloro-3-methoxybenzaldehyde and both product oils were combined to give 4.37 g (86%) of dimethyl acetal 3.

IR (neat, cm^{-1}): 2930, 2810, 1582, 1580, 1483, 1460, 1402, 1348, 1268, 1100, 1059, 989, 861, 827, 790

^1H NMR (CDCl_3 , ppm): 3.30 (6H, s), 3.90 (3H, s), 5.33 (1H, s), 6.95 (1H, d), 7.03 (1H, s), 7.32 (1H, d)

Diethyl 1-methoxy-1-(4-chloro-3-methoxyphenyl)methane phosphonate 4

[0031] A solution of dimethyl acetal 3 (4.3 g, 20 mmol), sieve-dried CH_2Cl_2 (20 ml) and triethyl phosphite (4.1 ml, 24 mmol) was stirred under argon at -78°C . Boron trifluoride etherate (2.95 ml, 24 mmol) was added dropwise at -78°C , the solution was stirred 5 min and stored overnight at -20°C . The next day the reaction was warmed to room temperature and stirred 5 hours to complete phosphonate formation. With vigorous stirring, the reaction was quenched with solid NaHCO_3 followed by 40 ml saturated NaHCO_3 solution. After gas evolution ceased, 40 ml CH_2Cl_2 and 20 ml H_2O were added, the biphasic mixture was partitioned and the CH_2Cl_2 phase was recovered, dried over Na_2SO_4 and evaporated. After pumping in vacuo, the oil was purified on a silica gel plug, eluting with CH_2Cl_2 to yield phosphonate 4 as a light yellow oil (6.01 g, 99%).

IR (neat, cm^{-1}): 2980, 2930, 1590, 1580, 1480, 1460, 1408, 1280, 1250, 1095, 1055, 1025, 967, 871, 809, 790, 754, 728

4-Chloro-3-methoxy-1-(methoxy-5-chloro-tricyclo[3,3,1, $1^{3,7}$]dec-2-ylidenemethyl)-benzene 5

[0032] Phosphonate 4 (3.2 g, 10 mmol) was dissolved in 30 ml dry THF under argon and cooled to -78°C . Dropwise addition of nBuLi (2.3M, 4.4 ml, 10.1 mmol) generated a yellow-orange phosphonate ylid. After stirring the ylid solution for 10 min, 5-chloro-2-adamantanone (1.75 g, 9.5 mmol), dissolved in 8 ml THF, was added dropwise to the ylid at -78°C . The reaction was slowly warmed to room temperature over 45 min and refluxed for 2 h. Upon cooling, the THF was stripped in vacuo and the product was partitioned between EtOAc/hexanes (1:1) and dilute NaHCO_3 . The organic layer was dried over NaHCO_3 . The organic layer was dried over Na_2SO_4 , stripped of solvent and purified on silica gel (2-4% EtOAc/hexanes) to give 3.3 g (96%) of enol ether 5 as a colorless, viscous gum.

4-Chloro-3-hydroxy-1-(methoxy-5-chloro-tricyclo[3,3,1, $1^{3,7}$]dec-2-ylidenemethyl)-benzene 6

[0033] Demethylation to enol ether phenol 6 proceeded cleanly upon heating enol ether 5 (3.3 g, 9.3 mmol) in 22 ml DMF at 135°C in the presence of sodium ethanethiolate (14 mmol) for 1.5 h. The reaction was cooled and partitioned between 50 ml EtOAc, 100 ml 1M NH_4Cl and 10 ml saturated NaHCO_3 solution. The organic phase was recovered and washed well with water while the aqueous phase was washed once with EtOAc. The EtOAc layers were combined, washed with brine, dried over Na_2SO_4 and stripped of solvent in vacuo. The crude oil was purified on a silica gel column, eluting with 50% CH_2Cl_2 /hexanes, to give 3.6 g of phenol 6 as a crude oil. Further purification by two crystallizations from a cooled 15% CH_2Cl_2 /hexanes solution provided phenol 6 as a solid (2.18 g, 68%). IR (CHCl_3 , cm^{-1}): 3530 (OH), 3300 (OH), 2920, 2845, 1568, 1478, 1308, 1190, 1166, 1090, 1079, 1042, 1020, 821

^1H NMR (CDCl_3 , ppm): 1.57-2.28 (11H, m), 2.75 (1H, br s), 3.27 (3H, s), 3.41 (1H, br s), 5.57 (1H, s), 6.79 (1H, dd, $J=8$ Hz, 2 Hz), 6.93 (1H, d, $J=2$ Hz), 7.28 (1H, d, $J=8$ Hz)

Sodium 2-cyanoethyl 2-chloro-5-(methoxy-(5-chloro)tricyclo[3,3,1, $1^{3,7}$]dec-2-ylidenemethyl)-1-phenyl phosphate 7

[0034] To a solution of phenol 6 (0.75 g 2.2 mmol), triethylamine (400 μl , 2.86 mmol) and anhydrous THF (8 ml) was added to 2-chloro-2-oxo-1,3,2-dioxaphospholane (Fluka, 240 μl , 2.6 mmol) at room temperature under argon. The reaction was stirred for 3 hours, during which time triethylammonium hydrochloride precipitated out. The reaction solution was pipetted off the precipitate with a cotton-tipped syringe under a strong flow of argon. The precipitate was rinsed several times with ether and the combined solution and rinses were evaporated in vacuo to give a foam, which was protected from exposure to moisture.

[0035] The foam was dissolved in anhydrous DMF (4 ml) and stirred with dry NaCN (140 mg, 2.8 mmol) at room

temperature for 24 h to form the β -cyanoethyl phosphate diester. The reaction mixture was pumped at high vacuum at 55°C to remove DMF, leaving phosphate diester 7 as a gum. The crude phosphate diester was photooxygenated without further purification.

Syn- and Anti- disodium 2-chloro-5-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro-)tricyclo[3,3,1,1^{3,7}]-decan]-4-yl)-1-phenyl phosphate 1

[0036] Phosphate diester 7 was dissolved in 20 ml 10% MeOH/CHCl₃ to which was added 5,10,15,20-tetraphenyl-21*H*,23*H*-porphine (TPP, 0.8 ml of a 2 mg/ml CHCl₃ solution). The reaction mixture was saturated with oxygen and irradiated with a 250W, high pressure sodium vapor lamp wrapped with Kapton film at 5°C while passing oxygen through the solution. Analytical reverse phase HPLC analysis showed complete dioxetane formation upon irradiating 20 min. The solvents were stripped in vacuo at room temperature, the residue was pumped to a gum under high vacuum, and stored at -20°C.

[0037] The crude cyanoethyl phosphate diester dioxetane 8, dissolved in 11 ml MeOH, was deprotected with sodium methoxide (0.5 ml of 25%, weight NaOMe in MeOH) at room temperature for 30 min. Upon completion of β -elimination of the cyanoethyl group, 2 ml saturated NaHCO₃ solution was added and the MeOH was rotary evaporated. HPLC grade water (15 ml) was added and the brown solution was passed through a 0.45 μ nylon filter. The solution volume was adjusted to 40 ml with HPLC grade water and purified by preparative HPLC, using a CH₃CN/H₂O gradient through a polystyrene column (PLRP-S, Polymer Laboratories). The freeze-dried fractions yielded 0.81 g (74% from phenol 6) of dioxetane 1. Analytical reverse phase HPLC on a polystyrene column using a gradient of acetonitrile and 0.1% NaHCO₃ and UV detection at 270 nm, showed one peak with a front running shoulder which represented a mixture syn and anti isomers. A sample of the product, as an isomer mixture, was dissolved in a 0.1 M diethanolamine buffer (1 mM MgCl₂) at pH 10. Upon being treated with alkaline phosphatase, light was emitted as expected, thus confirming the product as a 1,2-dioxetane of the entitled structure.

EXAMPLES

[0038] Various dioxetanes within the scope of this invention have been prepared and tested for essential properties. Included as prepared and tested dioxetanes are those where R is methyl, X is phosphate and Y² is chlorine, and the Z is at the 4 or 5 position on a phenyl ring. In the tests below, those dioxetanes are compared against commercial standards CSPD® and AMPPD™.

Chemiluminescent Detection of Alkaline Phosphatase In Solution

[0039] Alkaline phosphatase (5.25 X 10⁻¹⁷ moles) was incubated in 0.5 ml of 0.1 M diethanolamine, 1 mM MgCl₂, pH 10, containing 0.4 mM dioxetane at room temperature. The chemiluminescence (5 second integral) was measured in a Berthold LB952T luminometer at 5, 10, 20, 30, 44, 50 and 60 minutes. Figures 1 and 2 compare the performance of CSPD® and CDP-Star™. Figure 1 shows the comparison of CSPD® and CDP-Star™ plotted as relative light units (RLU) vs time. The average of three replicates are plotted. Figure 2 shows the results obtained from another set of samples containing 1 mg/ml of the chemiluminescence enhancing polymer polyvinylbenzyltributylammonium chloride.

Chemiluminescent Detection of Biotinylated pBR322-35mer on Nylon Membrane

[0040] Biotinylated pBR322 35-mer (13.1 pg in 1 μ l) was spotted onto a small piece of positively charged nylon membrane (Tropilon-Plus™). The membrane was dried completely and DNA was fixed to the membrane by UV cross-linking (120 mJ/cm²). The membrane was wetted with PBS and then incubated in Blocking Buffer (0.2% 1-Block™, 0.5% SDS in PBS) for 10 minutes, in streptavidin-alkaline phosphatase conjugate (Avidx-APT™, Tropix; diluted 1:5000 in Blocking Buffer) for 20 minutes, and washed once for 5 minutes with Blocking Buffer and three times for 5 minutes with Wash Buffer (0.5% SDS in PBS). Membranes were then washed twice for 5 minutes with Assay Buffer (0.1 M DEA, 1 mM MgCl₂, pH 10.0). Finally, membranes were trimmed and sealed in a small square of heat-sealable plastic with 40 μ l of 0.25 mM CSPD or CDP-Star (diluted in Assay Buffer). The sealed piece of membrane was immediately taped to a tube (pre-incubated at 22°C) and placed in a Turner Model 20e luminometer (Turner Designs, Inc. Mountain view, CA). Light emission was recorded every 5 minutes, for a period of 24 hrs at 22°C. Figure 3 is a plot of the chemiluminescence intensity (RLU) vs time for CSPD and CDP-Star.

Chemiluminescent Detection of Western Blotted Human Transferrin

[0041] Purified human transferrin (Boehringer/Mannheim cat #1317 415) was serially diluted with 1X SDS-PAGE

loading buffer (0.06 M Tris-HCl, pH 6.8, 2.25% glycerol, 0.5% β -mercaptoethanol, 2% SDS, 0.05% bromophenol blue). Dilutions were heated at 95°C for 5 minutes and 5 μ l of diluted sample was loaded per gel lane. Samples were electrophoretically separated by SDS-PAGE on 10% polyacrylamide minigels, using a Hoefer SE250 minigel apparatus. Each blot contains 10, 3.3, 1.1, 0.37, 0.12 and 0.04 ng amounts of transferrin. Following electrophoresis, gels and membranes were equilibrated with transfer buffer (5 mM MOPS pH 7.5, 2 mM sodium acetate, 20% MeOH) for 15 minutes. Protein was transferred to PVDF (Tropifluor™) and positively charged nylon (Tropilon-Plus™) membrane for 1 hr at 90V at 4°C. Blots were incubated in Blocking Buffer (BB-1 [0.2% 1-Block™, 0.1% Tween-20 in PBS] was used for PVDF and BB-2[3% 1-Block™, 0.1% Tween-20 in PBS] was used for nylon) for 30 minutes. Blots were then incubated with rabbit polyclonal antihuman transferrin (Boehringer/Mannheim cat #615 015; diluted 1:5000 in the appropriate Blocking Buffer) for 30 minutes, and then washed twice for 5 minutes (PVDF with BB-1, and nylon with Wash Buffer [0.1% Tween-20 in PBS]). Next, blots were incubated with goat anti-rabbit IgG alkaline phosphatase conjugate (Tropix; diluted 1:10,000 in the appropriate Blocking Buffer) for 30 minutes, and then washed twice for 5 minutes as above. Blots were then washed twice for 5 minutes in Assay Buffer (0.1 M DEA, 1 mM MgCl₂, pH 10.0). Finally, blots were incubated with 0.25 mM CSPD or CDP-Star (diluted in Assay Buffer) for 5 minutes. Blots were drained of excess substrate solution, placed in plastic report covers, and exposed to Kodak XAR-5 film. Results obtained on nylon and on PVDF membranes are shown in Figures 4-5.

Chemiluminescent Detection of a Single Copy Yeast Gene on Nylon Membrane

[0042] Total genomic DNA from the yeast *Saccharomyces cerevisiae* was digested with EcoR 1 and Bgl 11 restriction endonucleases. 5.0, 0.5, and 0.05 μ g quantities of each DNA digest were separated by electrophoresis in a horizontal 0.8% agarose 1X TBE gel. Following electrophoresis, the gel was soaked in 1.5 M NaCl, 0.5 M NaOH for 45 minutes to denature the DNA and then incubated in neutralization buffer (1 M Tris, 1.5 M NaCl, pH 7.4) for 45 minutes. DNA was transferred to positively charged nylon membranes (Tropilon-Plus™) by overnight capillary transfer using 20X SSC. The membranes were air dried and the DNA was UV cross linked to the membranes at 120 mJ/cm². A 1 kb Bgl 11 fragment, excised from the yeast gene RPB 1, was gel purified and biotinylated by a random priming reaction incorporating biotin-14-dCTP. The membranes were prehybridized for 30 minutes at 68°C in hybridization solution (7% SDS, 0.25 M Na₂PO₄, 1.0 mM EDTA), hybridized overnight at 68°C with 5 ml of fresh hybridization solution containing 5 ng/ml of denatured probe and then removed from the hybridization solution and washed as follows: twice for 5 minutes with 2X SSC, 1% SDS at room temperature; twice for 15 minutes in 0.1XSSC, 1% SDS at 68°C; and twice for 5 minutes in 1X SSC at room temperature. The membranes were then incubated for 10 minutes in blocking buffer (0.2% casein, 0.5% SDS, PBS), and 20 minutes with 1:5000 dilution AVIDx-AP™ in blocking buffer. They were then washed in blocking buffer for 5 minutes, three times for 10 minutes in wash buffer (0.5% SDS, PBS), and twice for 2 minutes in assay buffer (0.1 M diethanolamine, 1.0 mM MgCl₂, pH 10.0) followed by incubation for 5 minutes in 0.25 mM dioxetane in assay buffer. After draining excess substrate solution, the membranes were wrapped in plastic and exposed to X-ray film. 60 minute exposures taken 10 minutes, 70 minutes, and 19 hours respectively after substrate incubation are reflected in the following exposures. Comparisons of CSPD and CDP-Star are shown in Figures 6, 7 and 8.

Chemiluminescent Detection of DNA Sequence Ladders

[0043] DNA sequencing reactions were performed with the Tropix SEQ-light™ kit using biotinylated (-20) universal primer and single stranded M13 mp18 template DNA. Reaction products were separated on a 6% polyacrylamide 8 M urea gel, transferred to Tropilon-Plus™ nylon membranes by capillary action, and UV crosslinked to the membrane (total irradiation - 120mJ/cm²). Chemiluminescent detection was performed by incubating the membrane for 10 minutes in blocking buffer (0.2% i-Block™, 0.5% SDS, PBS), for 20 minutes in conjugate solution (1/5000 dilution of Avidx-AP streptavidin alkaline phosphatase conjugate in blocking buffer), then washing 1X5 minutes with blocking buffer, 3X5 minutes with wash buffer (0.5% SDS, PBS), and 2X2 minutes with assay buffer (0.1 M diethanolamine, 1 mM MgCl₂, pH 10). Each membrane strip was incubated for 5 minutes with either 0.25 mM CSPD or CDP-Star in assay buffer. All steps were performed at room temperature in a large heat sealed plastic bag with moderate shaking (140-170 rpm). Comparison of CSPD and CDP-Star at three time points is provided. The time after incubation with substrate and exposure time to Kodak XAR-5 x-ray film are indicated on Figure 9.

[0044] Additional testing reflects values such as quantum yield (performed by an independent laboratory according to the procedure listed below), $T_{1/2}$ and the emission wavelength maxima. These dioxetanes are identified by number, and in the tables following after the number, the identity of the substituent on the adamantyl ring, if any followed by the identity of the Z substituent is given. In the compounds tested, X is phosphate. Values for quantum yield and $T_{1/2}$ are obtained both for the dioxetane alone in 0.1 molar DEA, and in the presence of an enhancement agent, Sapphire II.

Protocol for Quantum Yields Determination

[0045] 500 μL of $3.2 \times 10^{-4}\text{M}$ solution of a dioxetane in 0.1M DEA, pH 10.0 was placed in a 12 x 75 mm tube, at 20°C. The solution was equilibrated to 20°C in a refrigerated water bath for 10 minutes. 2 μL of alkaline phosphatase suspension was added to the tube containing dioxetane and immediately vortexed for 1 sec and placed in the 20°C water bath. The tube was then placed in MGM Optocomp® I luminometer and the light signal was measured at 1 sec integration times. After the light signal was measured, the tube was placed back into the 20°C water bath and the measurement was repeated. The total counts for the dioxetane were determined from the intensity data. Total counts observed for a given concentration of dioxetane is the product of Photon Detection Efficiency (PDE) of the luminometer, the quantum yield of dioxetane and the number of molecules capable of emitting light (concentration of dephosphorylated dioxetanes). PDE for the MGM Optocomp I luminometer was determined to be 2.56×10^{-3} , measured with a Bioluminescence absolute standard and utilizing the known spectral response of the luminometer's PMT and the known emission spectrum of the dioxetanes. The quantum yield is calculated by dividing the total counts measured by the PDE and the concentration of the dioxetane.

Calculation of Half Life or Half Time to Steady State Light Emission

[0046] From the Turner luminometer readout, the maximum signal was measured. The maximum signal minus the Turner light unit readings at 30, 150, 300, or 600 second intervals was calculated and graphed vs. time in seconds. From the graphs, an exponential equation was calculated to determine the half life.

[0047] The half lives of the dioxetanes were also determined directly from the Turner luminometer printouts.

Emission Maxima

[0048] To 2 ml of a pH 10 solution of 0.4mM dioxetane, 0.1M diethanolamine, 1mM MgCl_2 was added $9.9 \times 10^{-11}\text{M}$ alkaline phosphatase. The solution was equilibrated 5 minutes in a Spex Fluorolog Fluorimeter and then scanned 5 times at 0.5 sec/nm for chemiluminescent emission. The chemiluminescence emission wavelength maximum was recorded.

Chemiluminescent DNA Sequencing

[0049] DNA sequencing with chemiluminescent detection was performed as described in the Tropix SEQ-Light™ protocol. Briefly, DNA sequencing reactions were initiated with biotinylated primers using M13 single stranded phage DNA as a template. The reactions were separated by 8 M urea denaturing PAGE, transferred horizontally to Tropix Plus nylon membrane by capillary action, and cross-linked to the membrane by exposure to UV light using a Spectronics SpectroLinker XL-1500 at 200 mJ/cm². The membranes were incubated with blocking buffer (0.2% I-Block™, 0.5% sodium dodecyl sulphate/SDS, in phosphate buffered saline/PBS [20 mM sodium phosphate, pH 7.2, 150 mM NaCl]) for 10 minutes, incubated with a 1/5000 dilution of Avidx-AP streptavidin-alkaline phosphatase in blocking buffer for 20 minutes, washed for 5 minutes in blocking buffer, washed 3 x 5 minutes with wash buffer (0.5% SDS, PBS), washed 2 x 5 minutes with assay buffer (0.1 M diethanolamine, 1mM MgCl_2 pH 10), and then incubated with dioxetane solution (either CSPD, 140-17 or 128-87 diluted to 0.25 mM in assay buffer) for 5 minutes. The membranes were drained, sealed in a plastic folder and exposed to Kodak XAR-5 X-ray film. For the dioxetane 128-87, the exposure time was 70 minutes and for 140-17, 80 minutes, both 65 minutes after substrate addition. For the comparison of dioxetane 128-87 versus CSPD, the membrane exposure time was 5 minutes after a 24 hour incubation with substrate. Figures 10 and 11. The details of this type of protocol are reflected in Tropix SEQ-Light™ DNA sequencing system, commercially available from Tropix, Inc. 0.1M DEA, pH 10, 25°C
Dioxetane concentration $3.7 \times 10^{-7}\text{M}$ to $6 \times 10^{-6}\text{M}$

Compound	Quantum Yield	T 1/2 (min)	λ_{em}
128-70 (H,5-Cl)	1.4×10^{-4}	35.55	471
128-87 (Cl, 5-Cl)	1.2×10^{-4}	9.03	470
140-20 (H, 5-OMe)	1.5×10^{-5}	1.55	476
140-17 (Cl, 5-OMe)	2.3×10^{-5}	1.09	475
140-62 (H, 6-OMe)	1.1×10^{-6}	2.4	490

(continued)

Compound	Quantum Yield	T 1/2 (min)	λ L em
140-73 (Cl, 6-OMe)	6.8×10^{-7}	2.0	487
AMPPD	5.2×10^{-5}	2.1	477
CSPD	5.2×10^{-5}	1.6	475

0.09M DEA + 0.1% Sapphire II, pH 9.95, 25°C
Dioxetane concentration 1.8×10^{-7} M to 6.1×10^{-9} M

Compound	Quantum Yield	T 1/2 (min)
128-70 (H,5-Cl)	5.2×10^{-2}	172
128-87 (Cl, 5-Cl)	3.5×10^{-2}	70.6
140-20 (H, 5-OMe)	2.4×10^{-3}	4.34
140-17 (Cl, 5-OMe)	1.9×10^{-3}	1.1
140-62 (H, 6-OMe)	3.8×10^{-5}	6.49
140-73 (Cl, 6-OMe)	5.5×10^{-5}	2.22
AMPPD	6.4×10^{-4}	9.2
CSPD	6×10^{-3}	3.5

[0050] To demonstrate positively the interaction of the dioxetane, or at least the excited-state emitter, with enhancement agents of the type known for use in connection with dioxetanes, the wavelength for the emission maximum was detected in the absence of any enhancement agent, in the presence of BDMQ, and on a nylon membrane. The data are set forth in the following table.

Emission Max. nm			
Dioxetane	No Addition	+ BDMQ	On Nylon
128-70	471	463	461
128-87	470	464	459
140-20	476	466	461
140-17	475	464	463
140-62	490	482	477
140-73	487	479	481

DOT BLOT ASSAYS

[0051] As noted above, the dioxetanes of this invention are suitable for use in dot blot assays. The dioxetanes synthesized according to the synthesis route described above were employed in dot blot assays. In confirmation of the absence of chemiluminescence of the dioxetanes bearing a Z substituent at the six position, it should be noted that Compound 140-62 gave a consistent absence of signal, or, under optimum conditions, a barely detectable signal. Similarly, the dioxetane with the methoxy substituent at the six position with a chlorine substituent on the adamantyl ring, 140-73, gave no signal in dot blot assay, again confirming the lack of chemiluminescent activity in six-substituted metaphosphate phenyl dioxetanes. Figures 12-21.

[0052] Nitrocellulose and nylon membranes were spotted with a biotinylated 35 base oligonucleotide probe. The probe was diluted in 1X SSC to yield a starting dilution of 210 pg. Successive 1:2 dilutions of the starting dilution were spotted on the membranes, 12 spots total. The membranes were dried, subjected to optimum U.V. crosslinking (120mJ/cm²), blocked for 30 minutes in blocking buffer (nitrocellulose: 0.2% I-Block, 0.1% Tween-20, 1X PBS; nylon: 0.2% I-Block, 0.5% SDS, 1X PBS), incubated 20 minutes in a 1/5000 dilution of streptavidin-alkaline phosphatase conjugate diluted in blocking buffer, and washed as follows: 1 x 5 minutes in blocking buffer, 3 x 5 minutes in 1X PBS, 0.3%

Tween-20 (nitrocellulose) or 3 x 5 minutes in 1X PBS, 0.5% SDS (nylon); 2 x 5 minutes in substrate buffer (0.1M diethanolamine, 0.1mM MgCl₂, pH 10); 1 x 5 minutes in a 1/20 dilution of Nitro-Block (Tropix, Inc. Bedford, MA) diluted in substrate buffer (Nitrocellulose Experiment Only); and 2 x 5 minutes in substrate buffer (Nitrocellulose Experiment Only). The membranes were incubated with 0.25mM dioxetane diluted in substrate buffer for 5 minutes. Several membranes in both the nitrocellulose and nylon experiments were incubated with 0.25 mg/ml Calfax DB-45, Calfax 10L-45 or Calsoft T-60 (Pilot Chemical Company, Los Angeles, CA), 1.0 mg/ml Tween-20, 1.0 mg/ml Nitro-Block, and 0.25 mM dioxetane diluted in substrate buffer for 5 minutes. These membranes were not subjected to a 1/20 dilution of Nitro-Block. The membranes were then exposed to x-ray film and developed.

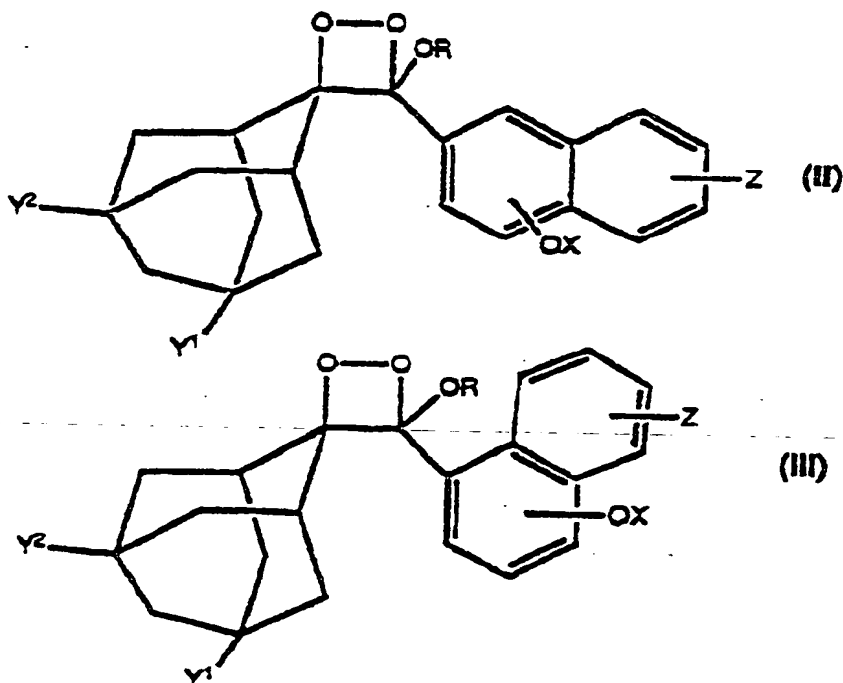
[0053] Thus, as can be seen from the results above, electron withdrawing groups added to the aromatic ring of the dioxetane alter the kinetics of light emissions while tending to increase the chemiluminescent signal. In contrast, electron-donating groups accelerate $T_{1/2}$ apparently by facilitating electron transfer from the oxygen, through the aromatic group, to the dioxetane. Thus, by proper selection of the nature and ability of the electron-donating or electron-withdrawing Z substituent, and simultaneous selection of the appropriate substituent for the adamantyl ring, if desired, dioxetanes of specific characteristics, including optimized signal intensity, optimized speed, specific emission wavelength, and the like, can be obtained.

[0054] These dioxetanes can be used for assays of all types in which an enzyme capable of cleaving the dioxetane is present alone or can be attached to one element of the ultimate complex which the analyte, if present, will form. Conventional assay formats are known to those of skill in the art, and are described in the patents set forth above in the Background of the Invention. Exemplary disclosure of suitable assays appears in U.S. Patent 5,112,960, and the same is incorporated herein by reference. The assay format, per se, save for the enhanced performance therein by the dioxetanes of this invention, does not constitute an aspect of the invention.

[0055] The dioxetanes of this invention, as well as the intermediates therefore, have been disclosed by reference to both generic description and specific embodiment. Additionally, dioxetane performance has been described generally, and exemplified. The examples are not intended as limiting, and should not be construed as such. Variations in substituent pattern, identity, and the like, consistent with the disclosure will occur to those of ordinary skill in the art. Such variations and modifications remain within the scope of the invention, save as excluded by the positive limitations set forth in the claims below.

Claims

1. A dioxetane of the formula (II) or (III):



wherein Y¹ and Y² are independently H, a hydroxyl group, a halogen, an unsubstituted C₁-C₁₂-alkyl group, a hydroxy C₁-C₁₂-alkyl group, a halo C₁-C₁₂-alkyl group, a phenyl group, a halo phenyl group, a C₁-C₁₂-alkoxyphenoxy group, a C₁-C₁₂-alkoxy phenyl group, a hydroxy C₁-C₁₂-alkoxy group, a cyano group, an amide group, a C₁-C₁₂-alkoxy group or a carboxyl group,

wherein R is C₁-C₁₂ alkyl, phenyl, naphthyl, phenyl C₁-C₁₂-alkyl or naphthyl C₁-C₁₂-alkyl,

wherein X is an enzyme-labile group selected from the group consisting of a phosphate, galactoside, acetate, 1-phospho-2,3-diacylglyceride, 1-thio-D-glucoside, adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, adenosine, α -D-glucoside, β -D-glucoside, β -D-glucuronide, α -D-mannoside, β -D-mannoside, β -D-fructofuranoside, β -glucosiduronate, p-toluenesulfonyl-L-arginine ester, and p-toluenesulfonyl-L-arginine amide,

and wherein Z is an electron-active group selected from the group consisting of Cl, OM, OAr, NM₃⁺, NHCOM, NMCOM¹, NHCOAr, NHCOOAr, NHCOOM, NMCOOM¹, CM₃, NO₂, COOM, COOAr, NHSO₂OM, NHSO₂Ar, CF₃, Ar, M, SiM₃, SiAr₃, SiArM₂, SO₂NHCOM, SO₂NHCOAr, SO₂M, SO₂Ar, SM and SAr, wherein M and M¹ are independently C₁₋₆ alkyl, and Ar is phenyl or naphthyl,

and wherein OX is substituted on the naphthyl ring such that the number of ring carbons between the point of substitution of the naphthyl ring on the dioxetane, including the carbon at that point of substitution, and the point of substitution of OX, including the carbon at that point of substitution, is an odd number,

and wherein Z is a substituent on any ring carbon save for those adjacent to the point of substitution on the dioxetane ring.

2. The dioxetane of claim 1, wherein Z is chloro, methoxy or amido substituents at the four, five or seven position.
3. A kit for conducting an assay employing a chemiluminescent dioxetane reporter molecule, comprising the dioxetane of claim 1 or 2, and an enzyme capable of cleaving, in aqueous solution, moiety X of said dioxetane.
4. The kit of claim 3, further comprising an enhancement substance for increasing the chemiluminescent signal obtained from said dioxetane upon cleavage of said X moiety in aqueous solution.
5. The kit of claim 3, wherein said assay is an immunoassay, and said enzyme is complexed with an agent capable of binding to an analyte, the presence or concentration of which said assay is conducted to detect.
6. The kit of claim 3, wherein said assay is a DNA probe assay, and said kit further comprises a membrane on which said assay may be conducted.
7. The kit of claim 6, further comprising an enhancement substance for increasing the chemiluminescent signal obtained from said dioxetane upon cleavage of said X moiety in aqueous solution.
8. The kit of claim 6, wherein said enzyme is complexed with an agent which in turn may be complexed with an analyte present in a sample, the presence or concentration of said analyte being that for which the assay is conducted.
9. The kit of claim 3, wherein said assay is a DNA sequence analysis assay, and said kit further comprises a membrane on which said sequence analysis assay may be conducted.
10. The kit of claim 9, wherein said kit further comprises an enhancement substance for increasing the chemiluminescent signal obtained from said dioxetane upon cleavage of said X moiety in aqueous solution.
11. The kit of claim 9, wherein said enzyme is complexed with an agent permitting attachment of the enzyme to the DNA to be sequenced in said assay.
12. A method of detecting the presence of an enzyme in a sample comprising contacting said sample with a dioxetane of claim 1, and detecting release of light caused thereby, wherein said enzyme cleaves said enzyme-labile group X and wherein detection of light released indicates presence of said enzyme in a sample.
13. The method of claim 12, wherein said X group is phosphate and said enzyme is alkaline phosphatase.
14. A method of detecting a first member of a specific binding pair having first and second members, comprising: optically detecting chemiluminescence produced by the reaction of a dioxetane of claim 1 or 2, with an enzyme

EP 1 120 422 A1

which cleaves said enzyme-labile group X of said dioxetane, wherein said enzyme is complexed with a second member of said specific binding pair.

15. The method of claim 14, wherein said method comprises an immunoassay.

16. The method of claim 14, wherein said method comprises a nucleic acid probe assay.

5

10

15

20

25

30

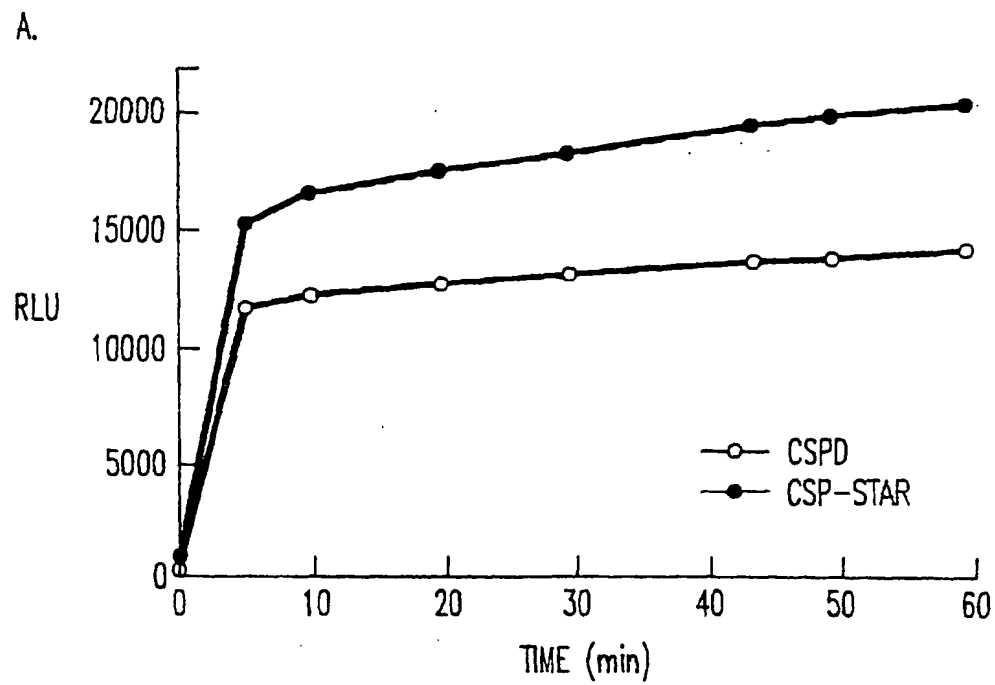
35

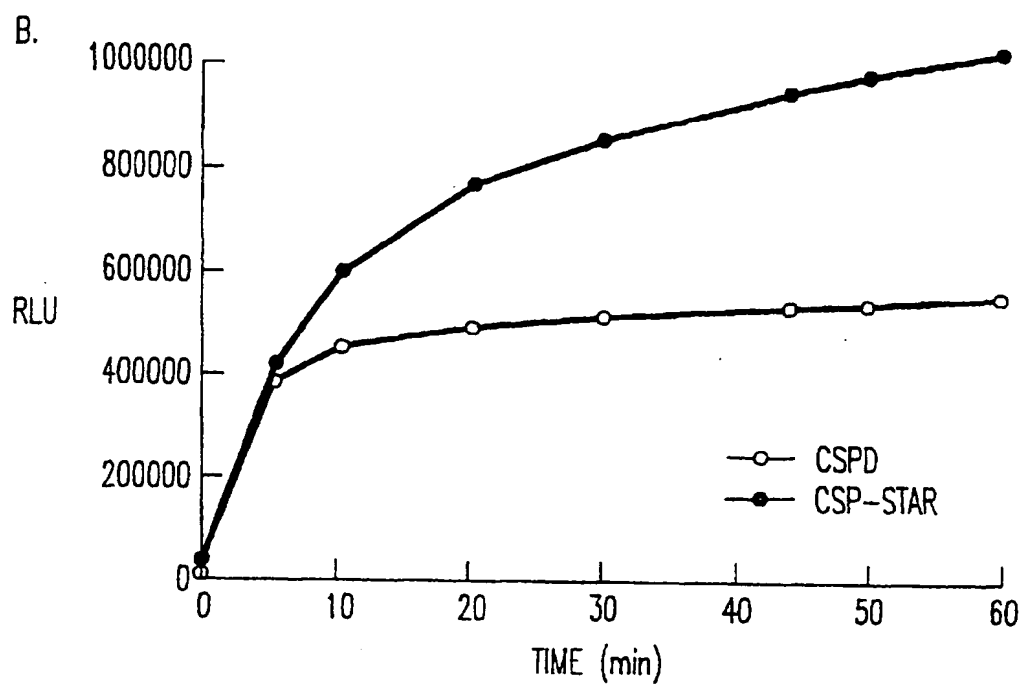
40

45

50

55

*FIG. 1*

*FIG. 2*

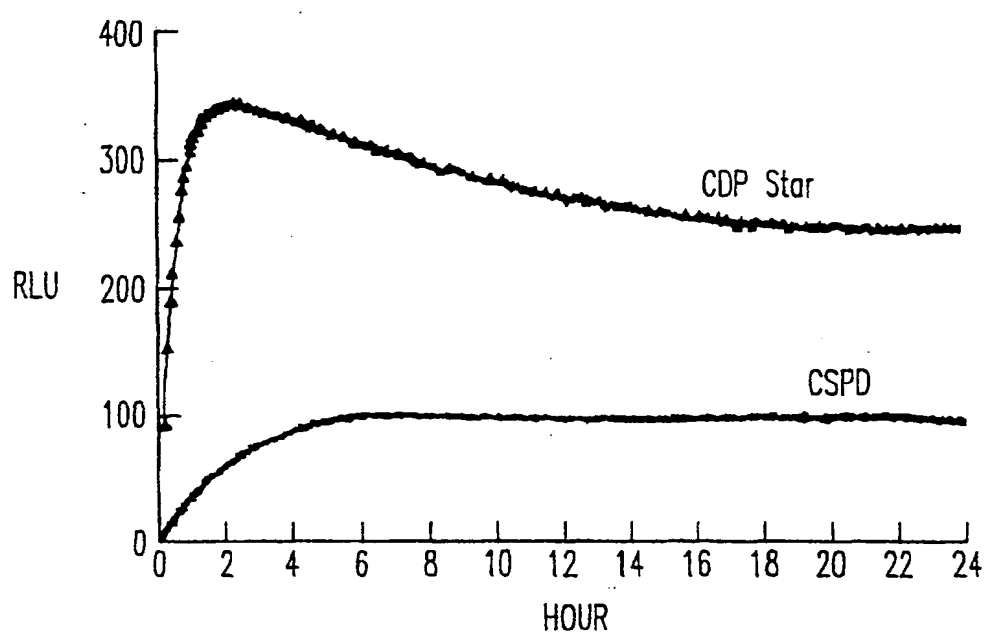


FIG. 3

CSPD®

**35 min inc.
2 min exp.**

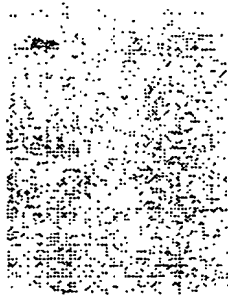


FIG. 4A

CDP-Star™



FIG. 4B

CSPD®

**19 hr inc.
30 sec exp.**

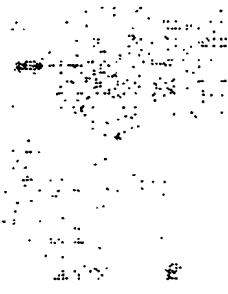


FIG. 4C

CDP-Star™



FIG. 4D

CSPD®

CDP-Star™

**10 min inc.
15 min exp.**

FIG. 5A

FIG. 5B

CSPD®

CDP-Star™

**55 min inc.
30 sec exp.**

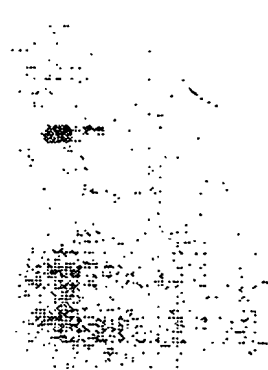
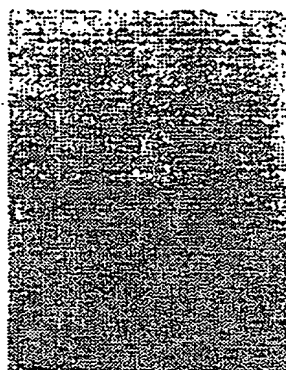
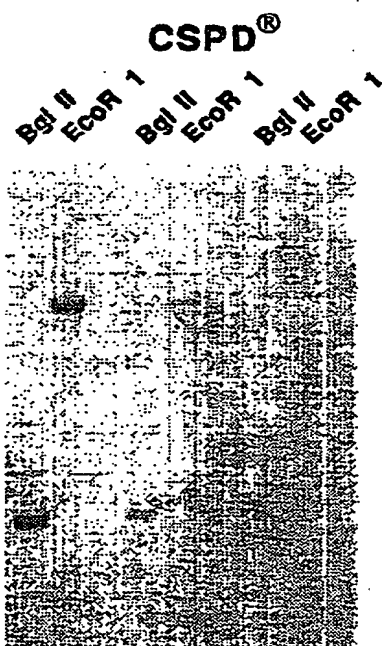


FIG. 5C

FIG. 5D

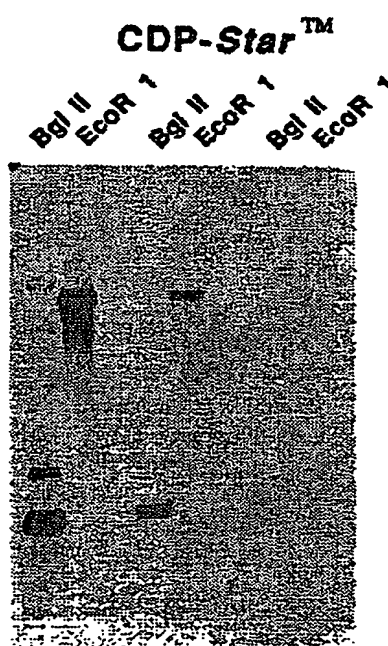
10 minute incubation
60 minute exposure



5.0 0.5 0.05

FIG. 6A

10 minute incubation
60 minute exposure



5.0 0.5 0.05 µg yeast DNA

FIG. 6B

70 minute incubation
60 minute exposure



FIG. 7A

70 minute incubation
60 minute exposure

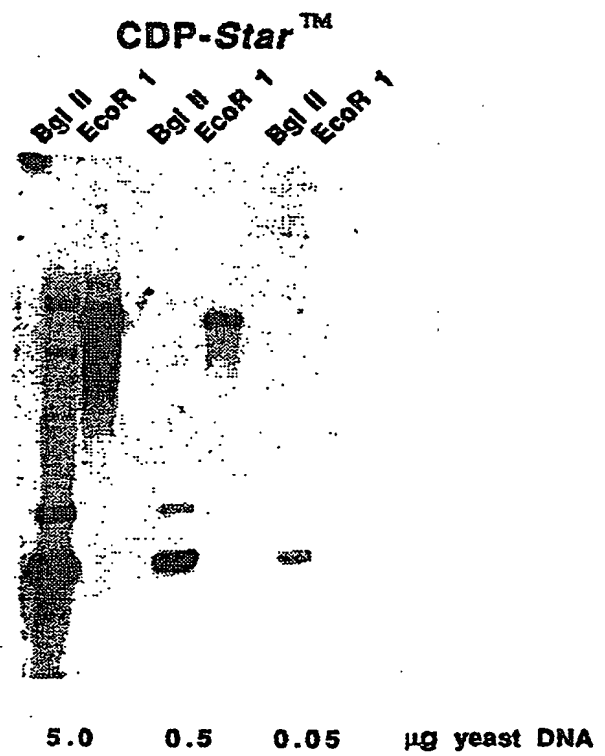


FIG. 7B

19 hour incubation
60 minute exposure

CSPD®
Bgl II EcoR I Bgl II EcoR I Bgl II EcoR I

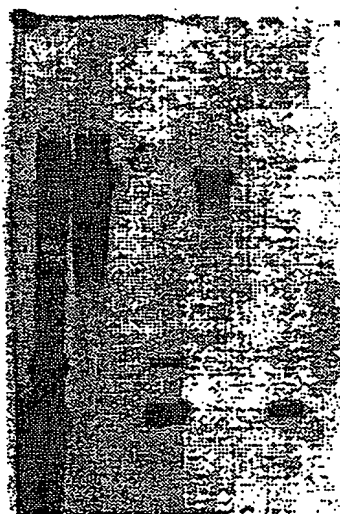


5.0 0.5 0.05

FIG. 8A

19 hour incubation
60 minute exposure

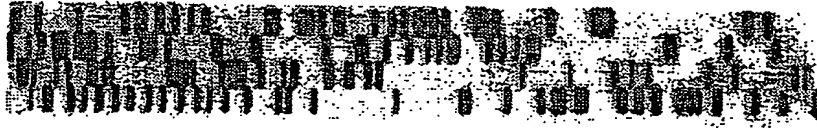
CDP-Star™
Bgl II EcoR I Bgl II EcoR I Bgl II EcoR I



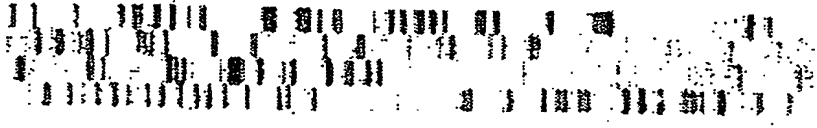
5.0 0.5 0.05 μ g yeast DNA

FIG. 8B

CDP-Star™



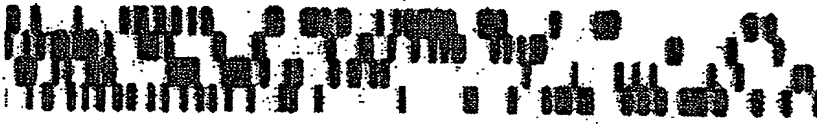
CSPD®



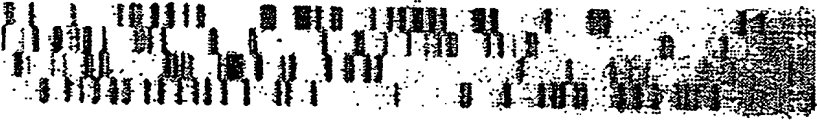
14.5 hour incubation
10 minute exposure

FIG. 9C

CDP-Star™



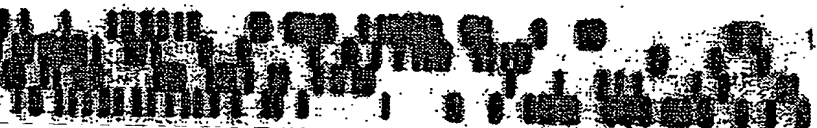
CSPD®



220 minute incubation
20 minute exposure

FIG. 9B

CDP-Star™



CSPD®



5 minute incubation
60 minute exposure

FIG. 9A

140-17

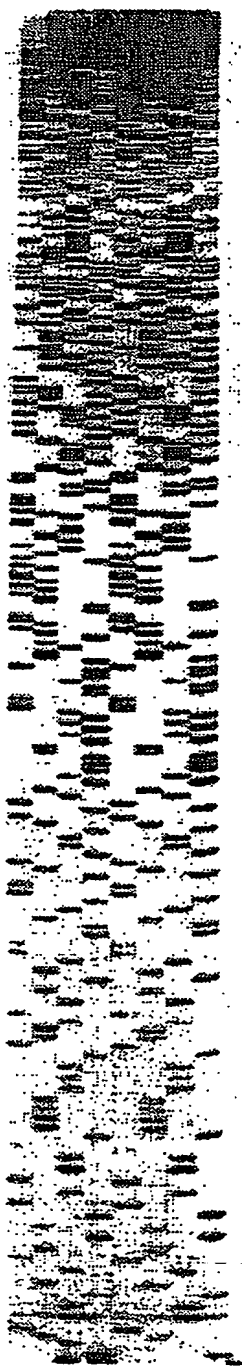


FIG. 10A

128-87



FIG. 10B

CSPD

128-87



FIG. 11A



FIG. 11B

30 min incubation
60 min exposure
Nitrocellulose
plus NitroBlock

FIG. 12A

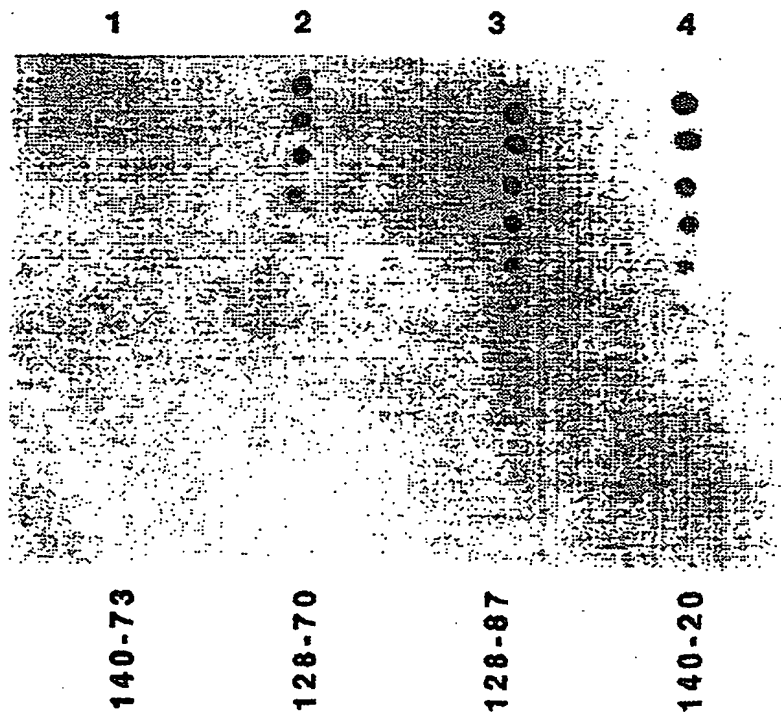
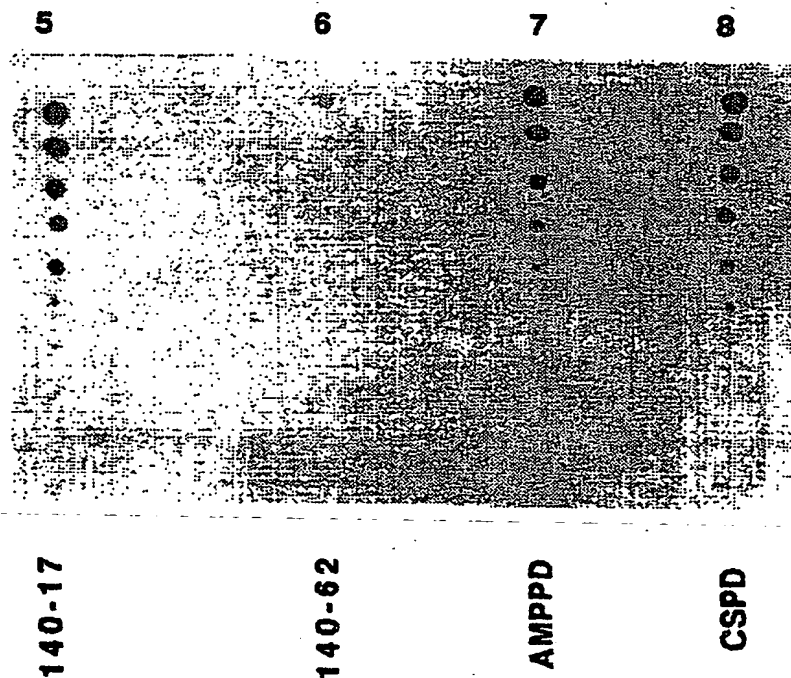
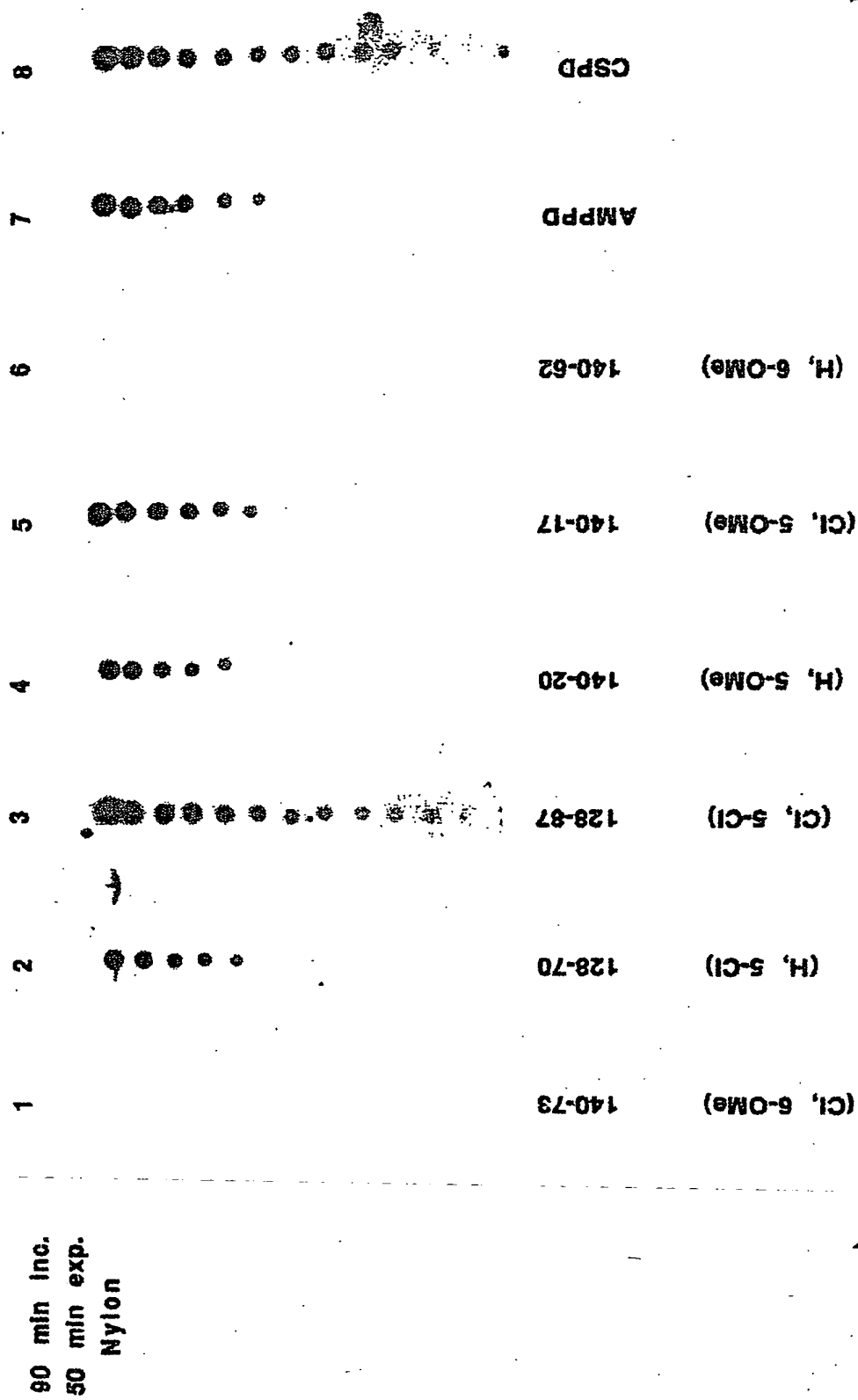


FIG. 12B





2.5 hour incubation
16 hour exposure
Nitrocellulose
(no NitroBlock)

FIG. 14A

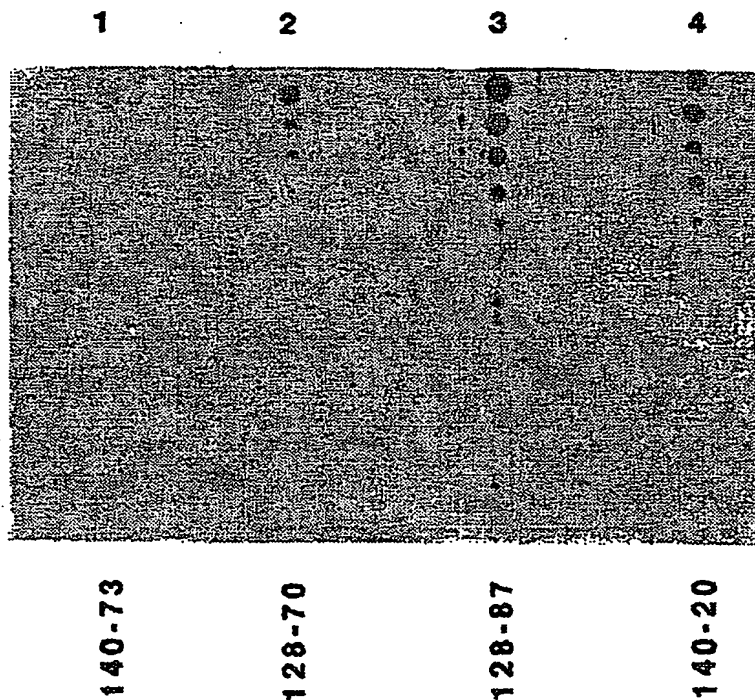
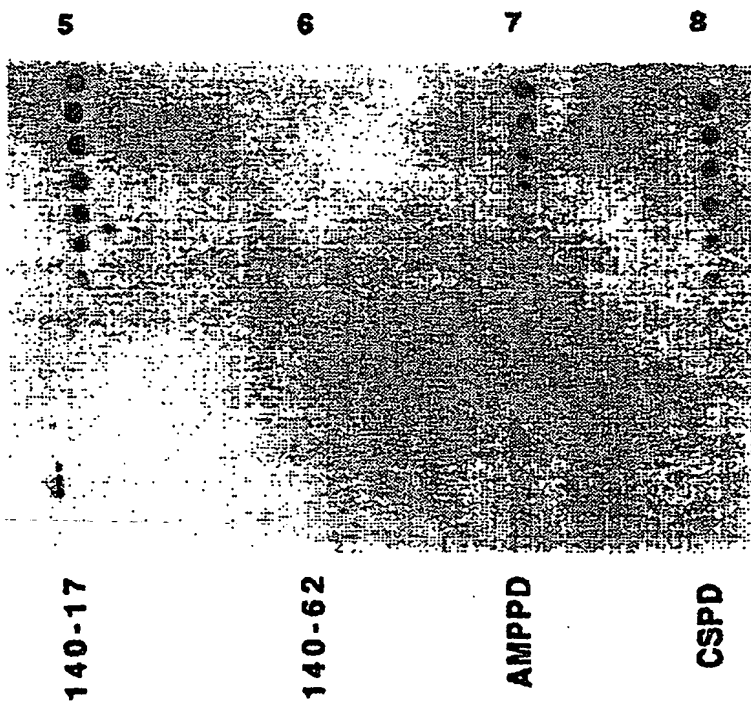
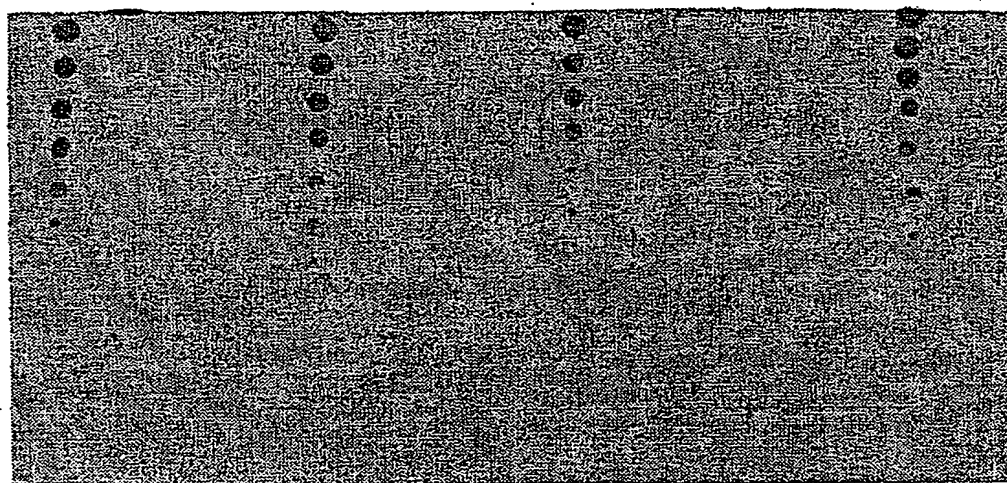


FIG. 14B





140-17

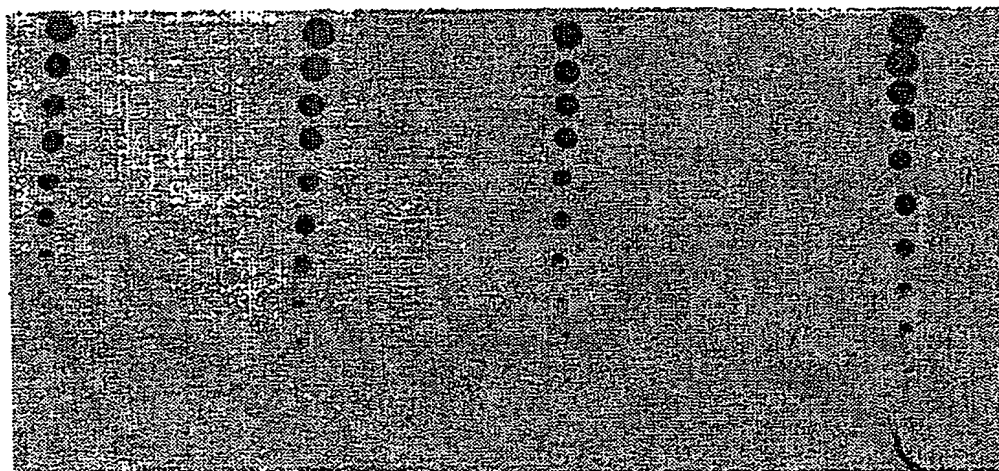
128-87

AMPPD

CSPD

NYLON
30 minute exposure
0 minute incubation

FIG. 15A



140-17

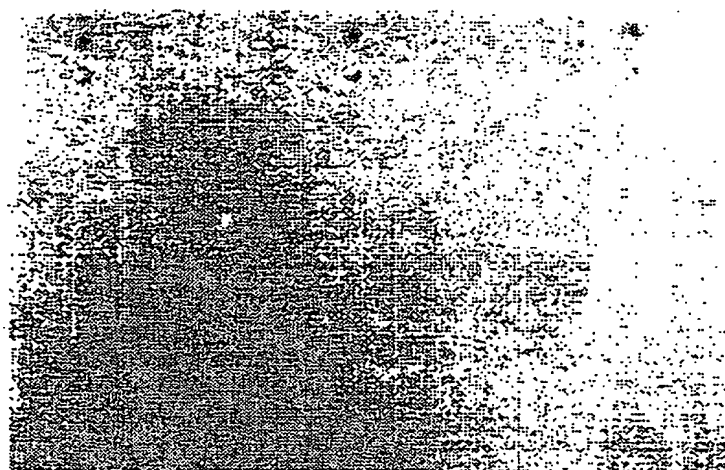
128-87

AMPPD

CSPD

NYLON
60 minute exposure
30 minute incubation

FIG. 15B



140-17

128-87

AMPPD

NITROCELLULOSE
30 minute exposure
0 minute incubation

FIG. 16A



140-17

128-87

AMPPD

NITROCELLULOSE
60 minute exposure
30 minute incubation

FIG. 16B

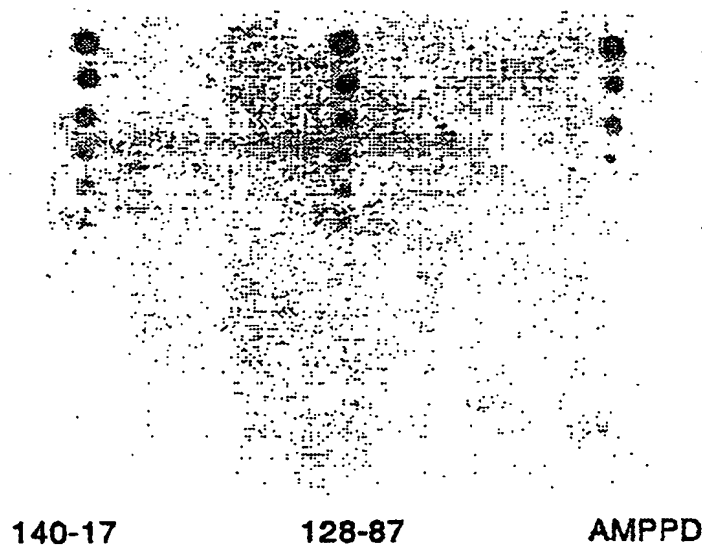


FIG. 17A

NITROCELLULOSE
120 minute exposure
60 minute incubation

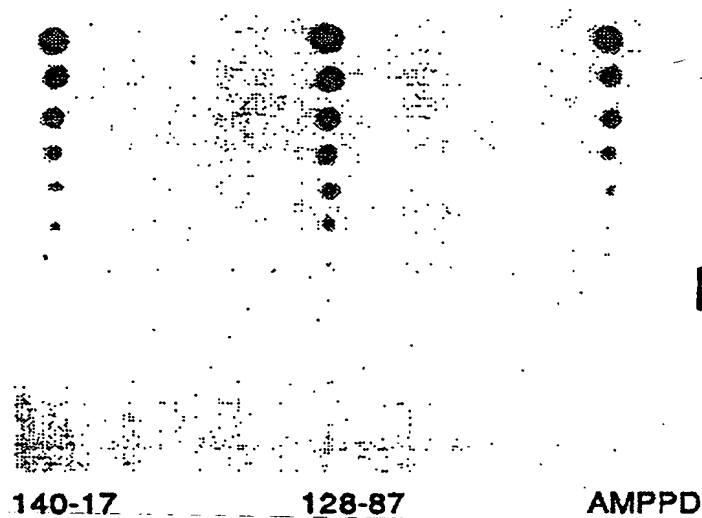
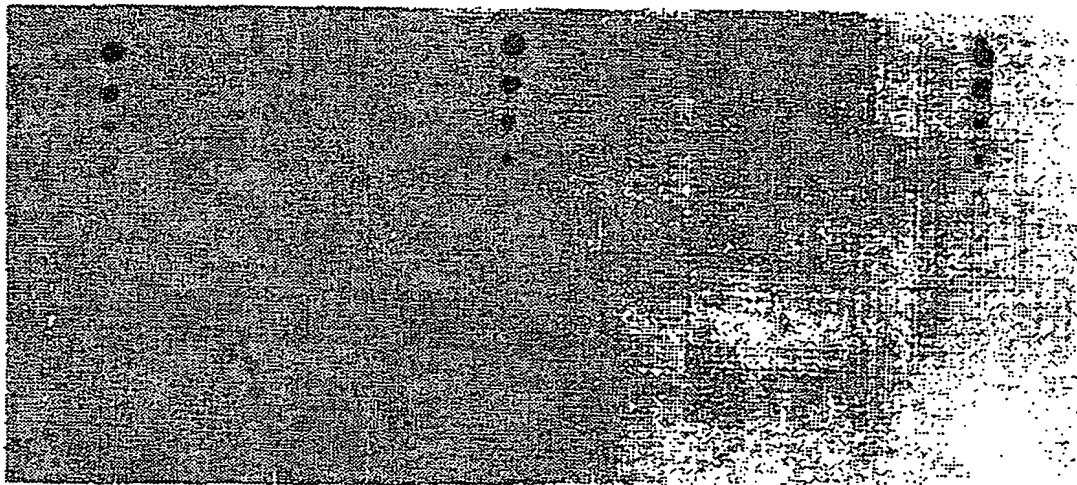


FIG. 17B

NITROCELLULOSE
15.5 hour exposure
2 hour incubation

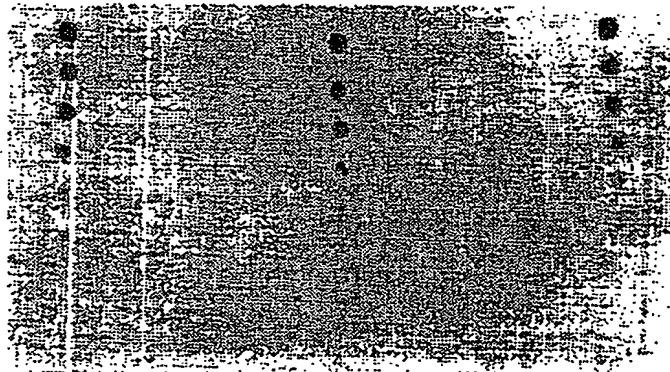


CSPD
Calfax DB45
30 min incubation
60 min exposure

CSPD
Calfax 10L-45
30 min incubation
60 min exposure

CSPD
Calsoft T-60
30 min incubation
60 min exposure

FIG. 18A

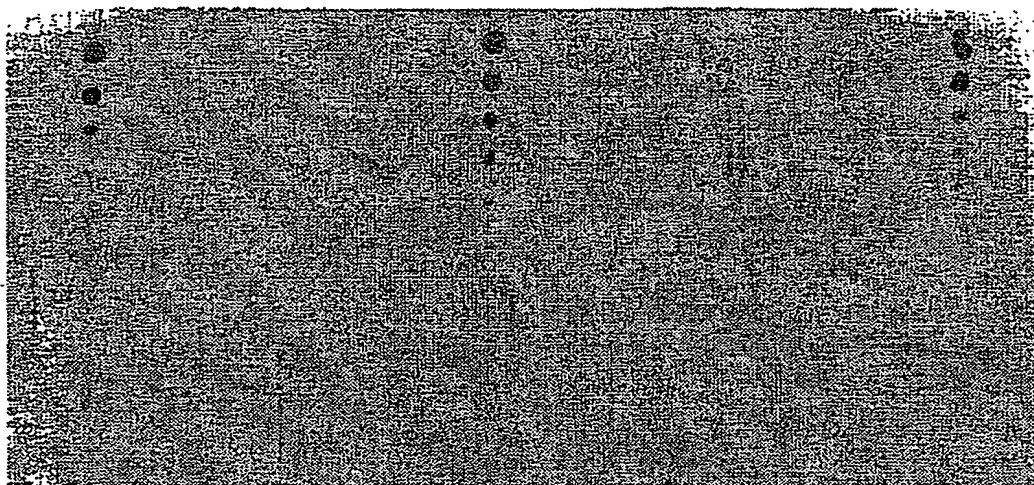


140-17
Calfax DB45
30 min incubation
60 min exposure

140-17
Calfax 10L-45
30 min incubation
60 min exposure

140-17
Calsoft T-60
30 min incubation
60 min exposure

FIG. 18B



CSPD
Calfax DB45
30 min incubation
60 min exposure

CSPD
Calfax 10L-45
30 min incubation
60 min exposure

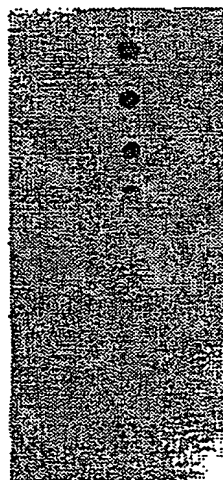
CSPD
Calsoft T-60
30 min incubation
60 min exposure

FIG. 19A



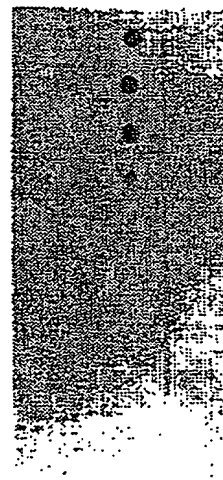
128-87
Calfax DB45
30 min incubation
60 min exposure

FIG. 19B



128-87
Calfax 10L-45
30 min incubation
60 min exposure

FIG. 19C



128-87
Calsoft T-60
30 min incubation
60 min exposure

FIG. 19D

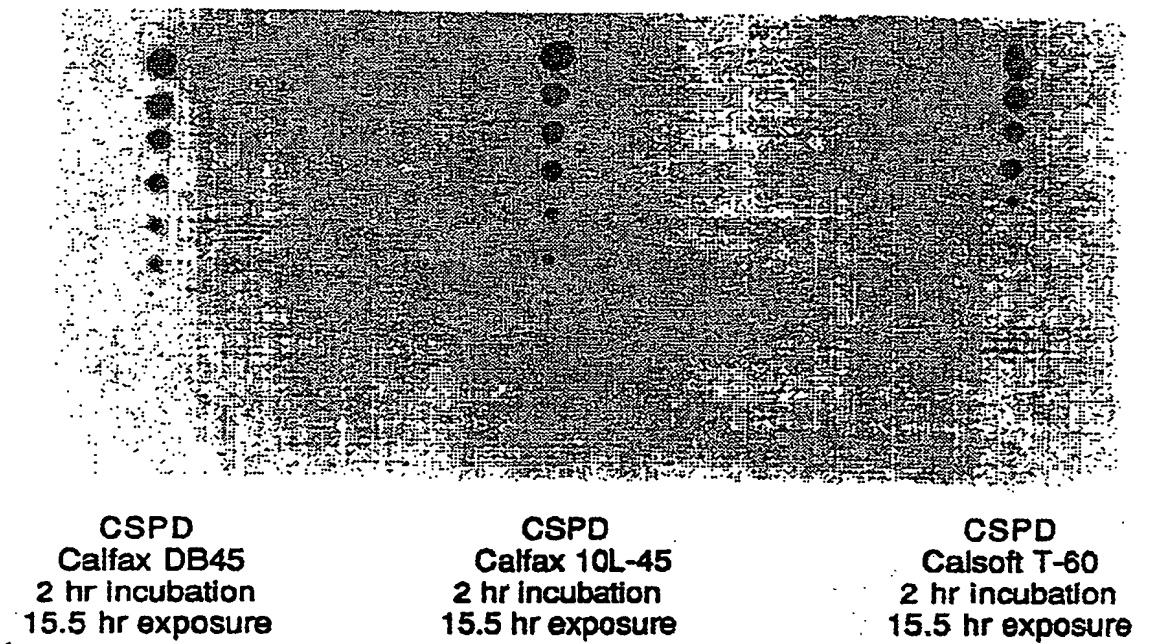


FIG. 20A

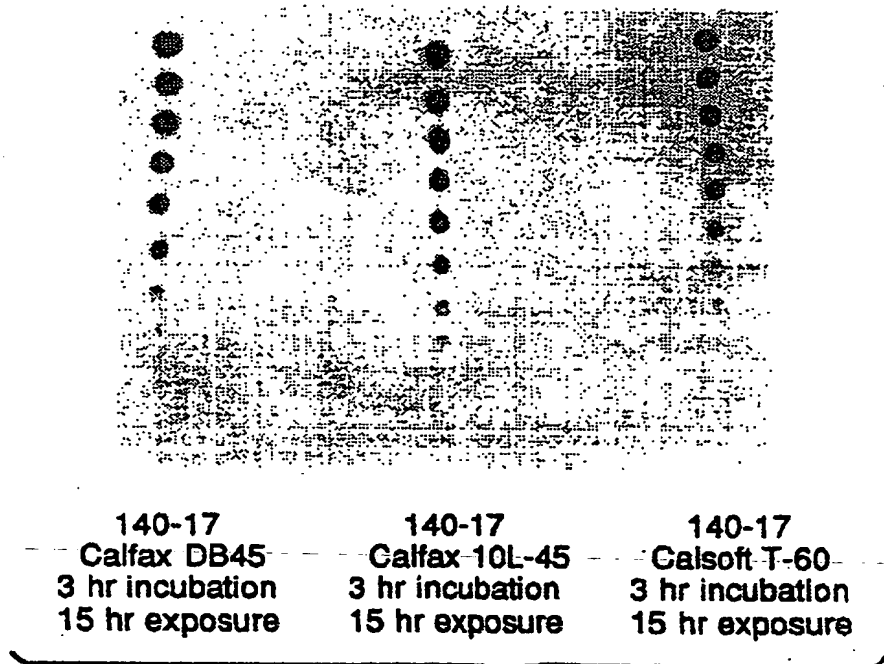


FIG. 20B

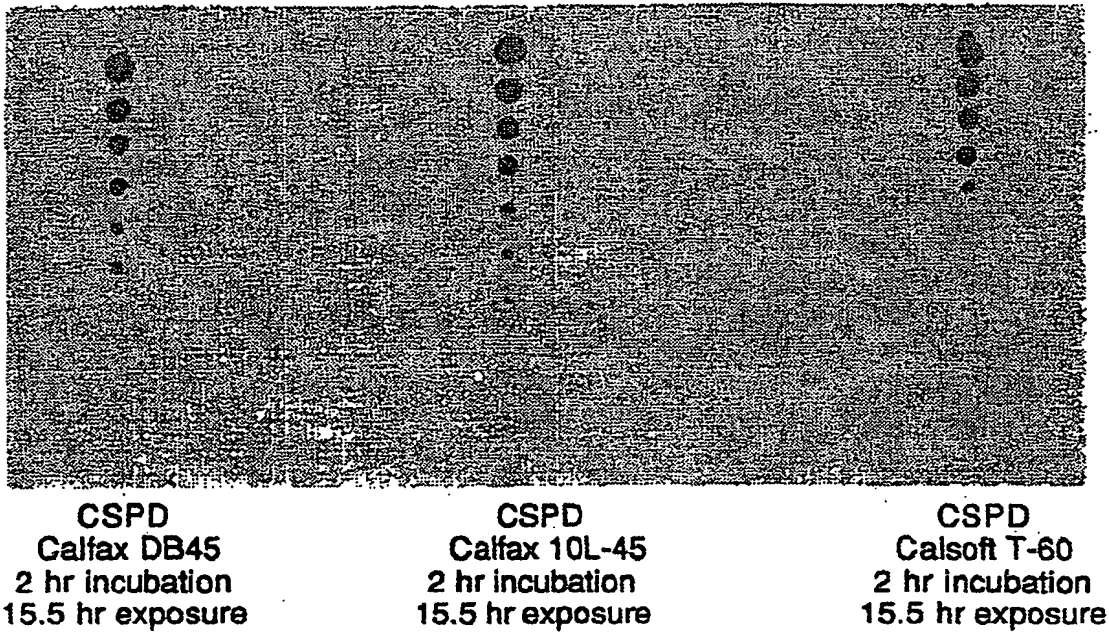
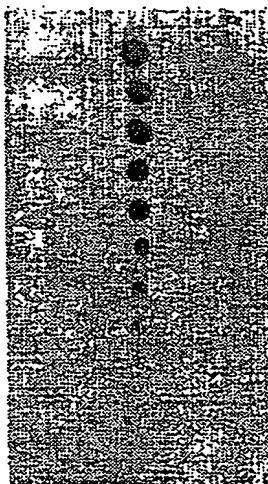


FIG. 21A



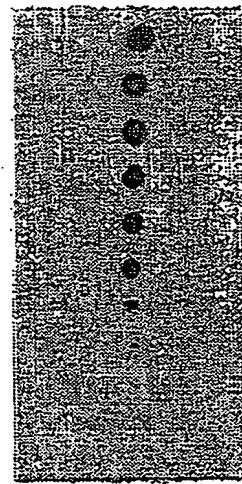
128-87
Calfax DB45
3 hr incubation
15 hr exposure

FIG. 21B



128-87
Calfax 10L-45
3 hr incubation
15 hr exposure

FIG. 21C



128-87
Calsoft T-60
3 hr incubation
15 hr exposure

FIG. 21D



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 01 10 4599

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	WO 92 04341 A (TROPIC INC.) 19 March 1992 (1992-03-19) * the whole document * -----	1-16	C07F9/655 C12Q1/42 C12Q1/34
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
			C07F C12Q
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 5 April 2001	Examiner Beslier, L
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

EPO FORM 1503 03/82 (P04011)

**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

EP 01 10 4599

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

05-04-2001

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9204341 A	19-03-1992	US 5112960 A	12-05-1992
		US 5220005 A	15-06-1993
		AT 174914 T	15-01-1999
		AU 656927 B	23-02-1995
		AU 8871491 A	30-03-1992
		BR 9105888 A	10-11-1992
		CA 2069957 A	01-03-1992
		DE 69130668 D	04-02-1999
		DE 69130668 T	20-05-1999
		DK 497972 T	23-08-1999
		EP 0497972 A	12-08-1992
		ES 2128326 T	16-05-1999
		FI 921925 A	29-04-1992
		JP 11021285 A	26-01-1999
		JP 2552413 B	13-11-1996
		JP 5503714 T	17-06-1993
		KR 176234 B	20-03-1999
		NO 302120 B	26-01-1998
		OA 9659 A	15-05-1993
		US 5547836 A	20-08-1996
		US 5538847 A	23-07-1996
		US 5582980 A	10-12-1996
		US 5543295 A	06-08-1996
		US 5605795 A	25-02-1997
		US 6140495 A	31-10-2000
		US 5654154 A	05-08-1997
		US 5851771 A	22-12-1998
		US 5831102 A	03-11-1998
		US 5330900 A	19-07-1994
		US 5326882 A	05-07-1994
		US 5994073 A	30-11-1999
		US 6022964 A	08-02-2000

EPO FORM P0488

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☒ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.